UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TEHCNOLOGY

INSTITUE OF MOLECULAR AND CELL BIOLOGY

Effect of cryopreservation on mammalian ovarian cortex

Bachelor's thesis

12 EAP

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TARTU 2022

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The increasingly effective, yet often dangerous cancer treatments, namely chemotherapy and radiotherapy, were found to have a lasting effect on women's reproductive system, causing multiple complications regarding fertility. One of the ways to preserve fertility in female patients is using cryopreservation of ovarian tissue. This method has multiple advantages over the cryopreservation of embryo or oocyte; however, an appropriate protocol is yet to be developed. During this bachelor's thesis, bovine ovaries were used as a model to try to identify the potential effect cryopreservation, by means of vitrification, may have on ovarian cortex, using histological and immunofluorescent methods.

Key words: ovarian cortex, vitrification, bovine, cryopreservation

CERCS: B210 Histology, cytochemistry, histochemistry, tissue culture; B450 Development biology, teratology, ontogeny, embryology (human); B570 Obstetrics, gynecology, andrology, reproduction, sexuality

Külmsäilitamise mõju imetaja munasarja koorele

Üha tõhusamad, kuid küllalt ohtlikud vähiravid, nimelt keemiaravi ja kiiritusravi, jätavad naiste reproduktiivsüsteemile püsivat mõju, põhjustades mitmeid viljakusega seotud tüsistusi. Üks viise, millega saab säilitada nais patsientide viljakuse, on munasarjakoe külmsäilitamine. Sellel meetodil on mitmeid eeliseid embrüo või munaraku külmsäilitamise ees, kuid asjakohane protokoll tuleb veel välja töötada. Selle bakalaureusetöö raames kasutati veise munasarju kui mudelit selleks, et tuvastada klaasistamise teel külmsäilitamise mõju munasarja koorele, kasutades histoloogilisi ja immunofluorestseeruvaid meetodeid.

Märksõnad: ovarian cortex, vitrification, bovine, cryopreservation

CERCS: B210 Histoloogia, tsütokeemia, histokeemia, koekultuurid; B450 Arengubioloogia, teratoloogia, ontogenees, (inim)embrüoloogia; B570 Sünnitusabi, günekoloogia, androloogia, paljunemine, seksuaalsus

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Abbreviations

POF - premature ovarian failure

PFA - paraformaldehyde

ITS - insulin-transferrin-selenium

BSA – bovine serum albumin

PBS - phosphate-buffered saline

IVC – in vitro culture

Introduction

Women who are undergoing cancer treatment, specifically radiotherapy and chemotherapy, risk experiencing changes in their ovaries, such as follicle depletion and stromal damage, all of which may lead to early menopause and ovarian failure (Bedoschi *et al.*, 2016; Morgan *et al*., 2012).

There are several methods available to women looking for fertility preservation options, including cryopreservation of embryos, oocytes, or ovarian tissue (Kim *et al.*, 2021). Embryo cryopreservation has been used for a long time and is a well-established way of fertility preservation. This procedure is limited to patients with an available sperm donor (Riggs *et al*., 2010). Oocyte cryopreservation has also provided successful results like embryo freezing. Both methods, however, require time for ovarian stimulation and oocyte retrieval, something that is not an option for patients, who are unable to delay their treatment. These procedures are also not applicable to pre-pubertal girls (Oktay *et al.*, 2003).

For patients who cannot make use of the two established cryopreservation methods, ovarian tissue cryopreservation can be method of choice. There is no need to delay cancer treatment as the tissue can be gathered immediately, hormone stimulation is not needed and people with hormone responsive cancers can participate, and this is so far the only fertility preservation method for young pre-pubertal girls (Christianson *et al*., 2021, Resetkova *et al*., 2013). Ovarian tissues can be cryopreserved by a slow freezing method; however, it has been found to be a long and tedious procedure with a risk of ice crystal formation, which leads to tissue damage (Gastal *et al*., 2019). For these reasons, vitrification, a rapid freezing method, was introduced. Although new, vitrification of ovarian tissue has already proven to have better results in some criteria (Keros *et al*., 2009), but there is no standard procedure protocol. There are varying results with different animals and cryosolutions.

This thesis aims to identify effect of given cryopreservation protocol on follicle survival, tissue proliferation and apoptosis. The thesis was done in Competence Centre on Health Technologies AS.

1. LITERATURE OVERVIEW

1.1. Normal function of the ovaries

Ovaries are the female reproductive organs, where the main functions are the development and maturation of oocytes, the female gametes, as well as the production of sex hormones. In most mammals the gametes stop proliferating before birth, so a person is born with a fixed number of the primordial follicles (McGee and Hsueh, 2000; Adhikari and Liu, 2009). Each such follicle, residing in the cortex area of the ovary, consists of a primary oocyte in a dormant stage, arrested at meiosis I, and a flattened monolayer of granulosa cells. Primordial follicles make up the main stock of the follicle pool, in humans it is estimated that at birth a child has just under 1 million primordial follicles (Vanderhyden, 2002; Fauser and Heusden, 1997).

The recruitment of primordial follicles from their dormant state happens continuously throughout the entire life, though it is but a small part of the entire follicle pool. Most primordial follicles either remain in the quiescent state or gradually degrade (Adhikari and Liu, 2009). Those with the recruitment potential begin developing to primary follicles, the oocyte grows in size and flat granulosa cells transition to cuboidal. Then continues the development to secondary follicle, where the oocyte size still increases, and granulosa cells start to proliferate forming multiple layers around the oocyte. The following antral follicle stage involves the appearance of an antrum, a cavity filled with fluid, and the differentiation of some granulosa cells to cumulus cells (Vanderhyden, 2002; McGee and Hsueh 2000; Gougeon, 1996).

When a woman reaches puberty, cyclic recruitment of antral follicles begins, and oocytes exit their meiosis I arrest. Generally, during maturation, no more than one fully developed Graafian follicle forms in the ovary while other majority, which were also recruited to mature, undergo follicle atresia (McGee and Hsueh, 2000; Zhang and Liu, 2015). Such cycles of recruitment occur continuously until the ovarian reserve is exhausted and the woman reaches menopause (Hamish *et al*., 2010).

The oocyte development from follicle activation until ovulation is very carefully regulated by different endocrine and paracrine signaling. Primordial follicles can stay in a dormant state for a long time and it has been shown to be under the influence of the surrounding niche. Loss of

growing follicles in ovaries is associated with an increased activation of primordial follicles by the decrease of anti-Mullerian hormone which is expressed by granulosa cells of primary to small antral follicles and acts as an inhibitor of activation (Roness *et al*., 2013; Carlsson *et al*., 2006). Another important feature of proper follicle development is communication between granulosa cells and oocytes. It has been shown that, through the PI3K and mTOR signaling pathway, granulosa cells differentiate from flat to cuboidal which then leads to the growth of oocyte (Zhang *et al*., 2014; Zhang *et al*., 2021; Zhang and Liu, 2015). The signaling between somatic cells and oocytes is said to be bidirectional, meaning the oocyte also regulates multiple features of granulosa cells. After initiation of growth, oocytes promote the proliferation and organization of granulosa cells around them. Absence or damage of the oocyte results in reduced proliferation and spontaneous luteinization of surrounding somatic cells (Vanderhyden, 2002; El-Hayek *et al*., 2018; Cheng *et al.*, 2013; Vanderhyden *et al.*, 1992). Oocyte derived factors also promote secretion of steroids (McGee and Hsueh, 2000).

1.2. The effect of chemo - and radiotherapy on mammalian ovaries

Though there have been made advancements in survival rates of patients with cancer, there remains a problem with the side effects from cancer treatment (Birch *et al.,* 1988; Morgan *et al.,* 2012). It has been noted that patients suffer from a lower quality of life following chemo – or radiotherapy. High dosage cancer treatment has multiple long lasting physical and psychological effects. Premenopausal young women and prepubescent girls, who are receiving treatment, get severe complications with normal function of the ovaries, not because of cancer itself, but due to the toxicity of treatment (Bedoschi *et al.*, 2016; Morgan *et al*., 2012).

The exact mechanisms of how the treatment agents affect the function of ovaries on a molecular level is still not fully known, but the direct and indirect effect can be studied using markers, such as inhibin-B and anti-Müller hormone (AMH). The documented outcomes of cancer treatments on ovaries include diminished ovarian reserve, by damaging the primordial follicles directly or cells around them, damaged stromal tissue, increased follicular atresia and damaged vasculature (Anderson and Cameron, 2011; Bedoschi *et al.*, 2016).

1.2.1. Chemotherapy induced damage

It is known that anti-cancer chemotherapy has a toxic effect on the ovarian reserve. Each chemical has a different impact on either directly the follicles or on the granulosa cells. These chemicals interfere with the normal proliferation of cells and cause DNA inra- and interstrand crossovers, which hinder DNA synthesis and function (Madden and Keating, 2014). Although some cells activate their repair mechanisms, which fix caused damages and ensure the survival of some follicles, still most cells go into apoptosis, as the DNA damage remains unfixed. There are also chemicals that cause DNA cross links during replication or inhibit topoisomerase reactions and cause the accumulation of toxic radicals (Soleimani *et al*., 2011; Bedoschi *et al*., 2016).

Aside from DNA damage, which brings cells to apoptosis, there are other ways how treatments, which are toxic to reproductive organs, damage primordial follicles. The ovarian reserve depletion can occur due to an increased activation of primordial follicles, the number of primordial follicles starts to decrease. As the chemotherapy chemicals damage the growing follicles, the inhibition of primordial follicles is halted and they begin growing to compensate for the loss (Sklar *et al*., 2006).

1.2.2. Radiotherapy induced damage

Radiotherapy is the usage of different dosages of ionizing radiation with the intent to kill cancer cells and tumors. Patients who are treated with irradiation, either in pelvic or abdominal or also full body area, experience endocrine dysfunction and loss of follicles caused by direct DNA damage or accumulation of oxygen radicals, all of which results in ovarian failure (Wo and Viswanathan, 2009; Devine *et al*., 2012). Women can also go into premature menopause, as radiation supports the depletion of follicles alongside the natural decline which happens with ageing. In a study on two groups of women who have undergone radiotherapy it has been estimated that 2 Gy dose of irradiation is enough to diminish the primordial follicle pool by 50%, while is has also been proven that dosages resulting in premature ovarian failure vary depending on the patient's age. Less radiation is required to cause ovarian failure in older women (Wallace *et al*., 2003).

1.3. Fertility preservation

Advancements in cancer treatment have shown to have significant improvements in survival rate of patients in all age categories and thus there is an increased need for different methods on how to preserve fertility in premenopausal patients struggling with the aftermath of chemotherapy and radiotherapy (Sklar *et al.*, 2006). Even though there is still a chance that the woman may recover from POF (premature ovarian failure), it is still important to have the discussion of options for maintaining the ability to conceive a child, as well as the hormonal regulation (Resetkova *et al.*, 2013; Donnez *et al*., 2004; Rodriguez-Wallberg *et al.*, 2019). There are several options to help women preserve fertility – ovarian transposition, embryo cryopreservation, oocyte cryopreservation, ovarian tissue cryopreservation and using gonadotropin-releasing hormone as protective agent during chemotherapy. Currently, cryopreservation technique is preferred. While assessing the necessary means to preserve the functionality of ovaries, multiple factors, like the patients age, the type of cancer or the time of oncotherapy, should be taken into consideration.

1.3.1. Embryo cryopreservation

Embryo cryopreservation is one of the most widely used and preferred fertility preservation technique (Kim *et al*., 2021). The technique has been used in assisted reproductive medicine for over 20 years and has shown good results in pregnancy outcomes (Riggs *et al*., 2010). This method often requires ovarian stimulation with hormones to acquire oocytes for in vitro fertilization. It is possible to retrieve the oocyte without stimulation, during natural cycle, but this results in a significantly smaller chance of generating an embryo as there are less mature oocytes retrieved (Oktay *et al*., 2003). The protocol for stimulation, however, can be dangerous for women with hormone responsive malignancies, such as breast cancer (Prest *et al*., 2002). Therefore, another method should be considered. It should also be noted that this method should only be used on patients with reproductive capabilities, therefore embryo cryopreservation cannot be offered to prepubertal girls. This method is not suitable on patients who cannot delay cancer treatment (Oktay *et al.*, 2003).

1.3.2. Oocyte cryopreservation

Another widely used method of fertility preservation is the cryopreservation of oocytes. Instead of an embryo, an unfertilized oocyte is frozen and later transferred back into the body or an in vitro fertilization is performed. Much like embryo cryopreservation, the retrieval of mature oocytes often requires hormonal stimulation, thus it is not applicable to girls without reproductive capabilities and patients with hormone responsive cancers (Oktay *et al.*, 2003). Though in this case, the problem of absence of a sperm donor is eliminated, as the oocyte is frozen without insemination, while also overcoming the problem of ethical restrictions in some countries regarding embryo cryopreservation. The issue with this method arises with the sensitivity of gametes to cryoprotectants and freezing procedure, damaging their cellular structures, and impairing the fertilization ability. Slow-freezing technique has been predominantly used to freeze oocytes for a longer time, but recently more attention has been drawn to vitrification as it yields better oocyte survival rate results (Kim *et al.*, 2021). Aside from mature oocytes, it is also possible to cryopreserve immature oocytes, those which haven't reached the metaphase II stage, and preform in vitro maturation, this allows to gather more oocytes that could be later used for fertilization (Lee *et al.*, 2013). Oocyte cryopreservation, however, still doesn't help patients who cannot delay their cancer treatment or prepubertal patients who are unsuitable for hormonal stimulation.

1.3.3. Ovarian tissue cryopreservation

It was first reported in 1994 of a successful transplantation of ovarian tissue after cryopreservation which resulted in fertility restoration. Even though the work was done on sheep, this had great potential in the field of assisted reproductive technology in humans (Gosden *et al*., 1994). Soon after there appeared first records of live birth in women using slow freezing – thawing technique (Donnez *et al*., 2004). This method of fertility preservation is the only option for prepubescent girls, patients who are at a higher risk when delaying treatment and who have hormone responsive tumors facing complications if they are to go under hormonal stimulation (Christianson *et al*., 2021, Resetkova *et al*., 2013). Either an oophorectomy or a minimally invasive biopsy is preformed to gather necessary tissue, depending on available

material. Collected tissue can range from a few fragments to a whole ovary. Generally, attention is drawn to grafting the cortex area of the ovaries as they contain many primordial follicles, therefore the method of cryopreservation of the ovarian cortex has an advantage in the number of fertilizable gametes after thawing. After gathering the ovarian tissues, they are treated with cryoprotectants and frozen in liquid nitrogen. The tissues can be stored in low temperatures for multiple years, so storage time varies depending on when the patients wish to have them reimplanted. Once the woman is ready, the tissue is thawed and reimplanted into the ovaries of the patient. In some cases, where there may be a risk of invasion of malignant cells, follicles are isolated from thawed tissues and cultured *in vitro* (Resetkova *et al*., 2013; Donnez *et al*., 2011; Amorim *et al*., 2009). Cryopreservation of ovarian tissue also provides hormonal preservation, meaning that to some extent the ovaries maintain their endocrine function and menstrual cycles. This gives the patient more freedom to choose when and how to get pregnant (Donnez *et al*., 2004; *Donnez et al*., 2011). Over forty children have been born using the ovarian tissue cryopreservation technique (Shapira *et al*., 2020).

1.4. Ways of tissue cryopreservation

1.4.1. Slow freezing

The first attempts at cryopreserving ovarian tissues were done by using controlled slow freezing when tissue pieces are frozen by gradually decreasing the temperature. This method was used widely and often yielded successful results in many experiments (Zhou *et al*., 2016). This technique however takes a longer time and poses a risk of ice crystal formation. Intracellular and extracellular ice crystallization can cause severe damages to the cellular structure of the tissue, either mechanical or by dehydration. Ice inside the cells brings about irreversible disruptions in normal functioning and ice outside of the cells causes shrinking by dehydration, all of which affect the cell survival (Fuller and Paynter, 2004). In addition, slow freezing requires a longer time and expensive machinery to freeze the tissue pieces, which lead to interests in a method that doesn't require a lengthy freezing procedure and an extensive machinery (Gastal *et al*., 2019).

1.4.2. Vitrification

Because of the problems found with slow freezing of ovarian tissue, vitrification is becoming an increasingly popular method of tissue cryopreservation. It is a very rapid way of cooling the tissue by directly plunging it into liquid nitrogen and avoiding the formation of ice crystals, but for such rapid changes to occur with minimal damage the amount of cryoprotectants used is increased, which in turn can be toxic (Zhou *et al*., 2016; Amorim *et al*., 2011; Sugishita *et al*., 2022). When freezing the tissue in a very low temperature it is crucial to ensure that no lethal damage occurs to the cells. To conserve the metabolic processes cryoprotecting agents are used (Raju *et al*., 2021). Main property of a cryoprotectant is its ability to form hydrogen bonds with water molecules and thus making it difficult for water to form ice crystals. Cryoprotectants are generally classified into two groups – the penetrating and non-penetrating agents. Penetrating cryoprotectants are smaller molecules able to diffuse through cellular membranes inside the cell and prevent the shrinkage caused by osmotic stress during freezing. Non-penetrating cryoprotectants include small and large polymeric molecules unable to permeate inside the cell but inhibit ice formation from the outside (Fuller, 2004; Bartolac *et al*., 2018).

Comparing controlled slow freezing with vitrification can be difficult, because of a lack in experiments with the latter and no universal protocol. As a result, many studies show contradicting results. For instance, for each method different results were seen in regards to apoptosis present in the ovarian tissue (Fabbri *et al.,* 2014; Zhou *et al*., 2010). In animal studies differences were observed in follicle morphology and vitality (Labrune *et al*., 2020; Vatanparast *et al*., 2018).

Tissue heterogeneity is the main problem when freezing the tissue because of different cell populations – stromal cells, granulosa cells and oocytes, have different penetrability and characteristics. It has proven challenging to find optimal rate of freezing and cryoprotectant concentrations for each cell type. When evaluation both freezing methods multiple parameters were found to be of concern – rapid activation of follicles, DNA damage, osmotic stress, or loss of hormone receptors (Lee *et al*., 2019). Vitrification is shown to be a superior method regarding preservation of stromal cells and overall viability of follicles (Keros *et al*., 2009; Herraiz *et al*., 2014).

2. Experimental part

2.1. Aim

The general aim of this thesis was to identify how does vitrification protocol affect the mammalian ovarian cortex. More specifically, the aims were:

- To determine the survival and morphology of follicles in ovarian tissue after vitrification procedure using histological methods.
- To determine if proliferation and apoptosis of the cells present in the ovarian cortex are affected by the vitrification procedure using immunofluorescent methods.

2.2. Materials and Methods

2.2.1. Preparation of bovine ovaries

The ovaries (n=4) of Bovines were collected from the Rakvere slaughterhouse and transported to Tartu where they were retrieved and put into a 50 ml tube (Greiner Bio-one, 227261) containing preequilibrated McCoys medium (ThermoFisher Scientific, 12330031) and put inside a 37^{o}C water bath (Lauda mgw CS6-D) until the processing. Ovaries were dissected in McCoys handling media supplemented with BSA (ThermoFisher Scientific, 12330031). The ovarian cortex was separated from the medulla and cut into 1 mm^3 pieces. Two replicates were immediately fixed in 4% PFA and Bouins for later use.

2.2.2. Culturing of ovarian cortex pieces

Fresh and vitrified cortical pieces were placed into culture medium for 6 days in a 37^{o}C and 5% CO_2 incubator (Eppendorf, Galaxy 170S). The culturing medium was αMEM (Sigma-Aldrich, M8042) supplemented with 0.1 mg/ml Penicillin-Streptomycin (ThermoFisher Scientific, 15140122), 1x Insulin-Transferrin-Selenium (ITS) solution (ThermoFisher Scientific, 41400045), 50 mg/ml L-ascorbic acid (Sigma-Aldrich, A92902), 10 mg/ml BSA (Sigma-Aldrich, A4503) and 3mM Glutamin (ThermoFisher Scientific, 35050061). Each well contained 1 cortex piece and 150 µl of incubated culture media and half of the culture media (75 µl) was changed every 2 days.

Following culture two replicates from the vitrified and fresh ovarian cortex were fixed in 4% PFA and Bouins.

2.2.3. Vitrification

Ovarian cortex pieces were vitrified using a protocol as previously described on Salehnia *et al*. First the Basic solution was prepared: 1 mg/ml of glucose (Sigma-Aldrich, G8769), 5.8 mg/ml of sodium pyruvate (Sigma-Aldrich, S8636), 0.05 mg/ml of penicillin-streptomycin (ThermoFisher Scientific,15140122) and 50 ml (ThermoFisher Scientific, 14190144), solution was filtered through a 0.2 µm sterile filter (Novatech international, FJ25BSCPS002AL01). Next the pre-vitrification solution was prepared: 25% of Ficoll PM 70 (Sigma-Aldrich, F2878), 0.73 M of sucrose (Sigma-Aldrich, 84100) and 8.8 ml of basic solution, the pre-vitrification solution was left overnight at room temperature so the cryoprotectants can dissolve properly. The ready-vitrification solution was prepared as follows: 2 ml of ethylene glycol (Serva, 11285.02), 0.5 ml (10 mg/ml) of BSA (Sigma-Aldrich, A4503) and 2.5 ml of pre-vitrification solution, the solution was filtered through 0.2 µm sterile filter (Novatech international, FJ25BSCPS002AL01).

Pieces of ovarian cortex were briefly incubated in ready-vitrification solution, which was preequilibrated at room temperature.

Immediately following incubation in cryoprotective solutions the fragments of the tissue were transferred to cryovials and immersed into liquid nitrogen, where they were stored for 6 days (Custom Biogenic Systems, CB601L).

2.2.4. Thawing

After removing samples from liquid nitrogen, the cryovials were held at room temperature in the air so the remaining liquid nitrogen could evaporate and then placed in 37^{o}C for 30 seconds.

For the removal of cryoprotectants from the tissue, the cortex pieces were thawed using decreasing sucrose (Sigma-Aldrich, 84100) concentrations (1M and 0.5M) diluted in basic solution – samples were incubated 2 minutes in the first warming solution containing 1M sucrose and 3 minutes in the second warming solution with 0.5M. Tissue pieces were then transferred for 5 minutes into washing medium containing BSA (Sigma-Aldrich, A4503), diluted in basic

solution, and finally transferred to pre-equilibrated McCoy's handling medium (ThermoFisher Scientific, 12330031) supplemented with 0.5% BSA (Sigma-Aldrich, A4503), 1% Glutamax (ThermoFisher Scientific, 35050061), 1% Penicillin-Streptomycin (ThermoFisher Scientific, 15140122) and 1% ITS (ThermoFisher Scientific, 41400045). Tissues were fixed in 4% PFA and Bouins or cultured for 6 days.

2.2.5. Histology

The tissue pieces fixed in Bouins and 4% PFA were serially dehydrated in graded ethanol, starting from 60% to 96%, and in xylene. Ovarian pieces were embedded in paraffin and 4 µm sections were obtained using microtome (ThermoFisher Scientific, Microm HM355S). Sections were collected in 37^{o}C water bath and transferred on adhesive slides (Ultra Plus and SuperFrost). All slides were placed at 40^{o}C overnight to dry.

2.2.5.1. Hematoxylin and eosin staining

Hematoxylin and eosin staining was performed on ovaries which have stained with Bouins for morphological analysis and follicle count, as well as follicle classification.

Hematoxylin and Eosin staining was performed per standard protocol. The stains are water-based and therefore not mixable with paraffin, the slides were firstly deparaffinized. Slides with sections were put on a 60^{o}C surface for the wax to melt and transferred in xylene for 5 minutes twice. Then the sections were serially rehydrated in graded ethanol until 50% and transferred to Milli-Q water. The slides were stained with hematoxylin for 30 seconds and rinsed with water. Then they were stained with eosin for 1 minute and rinsed with water. For dehydration the slides were quickly rinsed in 70% ethanol for about 5 seconds and transferred in 96% ethanol for 1 minute. Finally, the slides were immersed in xylene for 5 minutes twice and mounted with coverslips. Slides were then left overnight to dry.

The sections were assessed and evaluated under light microscopy (Nikon ECLIPSE TS100). Follicles were classified by their development based on granulosa cells as follows – primordial and primary follicles were counted as one group having a single layer of granulosa cells around an oocyte, secondary follicles were surrounded by two or more granulosa cells and antral follicles were surrounded by multiple layers of granulosa cells and had an antral cavity.

2.2.5.2. Immunofluorescence with Ki67

Samples fixed in 4% PFA were used for immunofluorescence using anti-mouse/rat ki67 antibody (ThermoFisher Scientific, 14-5698-82). This approach will help to identify cells within the tissue which are proliferated as the protein Ki-67 was found to be an important marker present in the nuclei of proliferating cells (Gerdes et al. 1984). The first day started with melting the wax on slides and rehydration. Slides were dipped into xylene twice for 5 minutes to remove wax and then were dehydrated through sequential 1-minute incubations with decreasing EtOH percentages (96%; 90%; 80%; 70%; 60% and 50%). Slides were finally washed in Milli-Q. For antigen retrieval, the slides were placed in a jar containing pre-warmed citrate buffer for 20 minutes. After cooling down, for better permeability of the antibody, 0.1% Triton-X, diluted in PBS, was added onto the slides, and incubated for 20 minutes and then the slides were washed with 1x PBS (ThermoFisher Scientific, 14190144) three times for 5 minutes. To block any non-specific antibody binding, the slides were incubated in 5% goat serum, diluted in 1% BSA in PBS, for 1 hour. After removing the goat serum from the slides, they were incubated in anti-mouse/rat ki67 primary antibody, 1:200 dilution in 1% BSA in PBS, overnight in the fridge in humidified chambers.

Day 2 started with washing the slides with PBS three times for 5 minutes each time. The slides were then incubated in the dark for 1 hour in secondary antibody Alexa 488, diluted in 1% BSA, and washed again with PBS for 5 minutes three times. Lastly the slides were incubated for 1 minute in a 1:1000 fluorescent stain DAPI, diluted in 1% BSA, and washed again with PBS for 5 minutes three times. The slides were mounted using fluoromount (Sigma-Aldrich, F4680) and after some time the sides of the slides were covered with clear nail polish to avoid air getting in. The stained slides were stored in 4 ^{o}C. Tissue pieces were visualized using a Fluorescence Microscope (Olympus, BX51) and analyzed in Fiji (software ImageJ2, version 2.35).

2.2.5.3. Immunofluorescence with TUNEL

The DeadEnd^{TM} Fluorometric TUNEL System (Promega, G3250) was used for the detection of apoptosis in the tissue cells. This system is used to detect DNA fragmentation in cells, as it is a common characteristic of apoptotic cells. The principal of the assay is labeling the 3’ -hydroxyl

terminus in DNA breaks with fluorescein deoxynucleotides using the recombinant terminal deoxynucleotidyl transferase enzyme.

The tissue sections were first deparaffinized by melting the wax and immersing the slides in xylene twice for 5 minutes each time. The slides were rehydrated using graded ethanol, up to 50%, washed with 0.85% NaCl for 5 minutes and PBS for 5 minutes. For better permeability of the tissue to staining reagents the slides were incubated with Proteinase K (included in the kit) for 10 minutes and then washed with PBS for 5 minutes three times. The tissue sections were fixed again by transferring 4% PFA onto them for 5 minutes and washing again with PBS for 5 minutes. The slides were equilibrated with equilibration buffer (included in the kit) for 10 minutes. From this step, the work was done in the dark as the fluorescein deoxynucleotides are light sensitive. Next, the slides were incubated with the incubation buffer (consisting of equilibration buffer, nucleotide mix and the rTdT enzyme, while the negative control buffer had the same components except instead of the enzyme, deionized water was added) for 1 hour in 37 ^{o}C chamber (Memmert) for the reaction to take place. After incubation the reaction was stopped by immersing the slides in 2X SSC solution for 15 minutes. Afterwards the slides were washed with PBS for 3 x 5 minutes and stained with 1:1000 DAPI, diluted in 1% BSA, for 2 minutes, washed with PBS 3 x 5 minutes. Lastly, the slides were mounted using fluoromount (Sigma-Aldrich, F4680) and kept in the dark. Tissue pieces were visualized using a Fluorescence Microscope (Olympus, BX51) and analyzed in Fiji (software ImageJ2, version 2.35).

2.2.6. Statistical analysis

The number intact follicles in different stages and cells emitting fluorescence were calculated in each group (fresh, fresh + 6 days of IVC, vitrified, vitrified +6 days of IVC). Calculations were done in GraphPad Prism 9.3.1. software and are presented as number of cells per tissue area. Fisher's test and Student t-test were used to evaluate statistical importance between groups. $P<0.05$ was considered to have statistical significance and $1>p>0.05$ was considered almost significant.

2.3. Results

2.3.1. Histological evaluation

A total of four bovine ovarian tissues were analyzed. Out of 262 follicles 199 (76%) were primordial/primary follicles and 63 (24%) secondary follicles, no antral follicles were observed (Table 1). Identified follicles were morphologically classified as intact, partially detached from granulosa cells, and degenerated (completely detached from granulosa cells and losing circular shape). There were no changes seen in the number of intact follicles in tissue are of 0.1 mm^2 between fresh and vitrified tissue pieces ($p>0.05$), although the difference between fresh and vitrified tissues that had gone through 6 days of in vitro culture (IVC) is nearing statistical difference ($p=0.1$). However, unvitrified fresh ovarian pieces had better results after 6 days of IVC, overall, there are more intact follicles ($p<0.05$), while in vitrified tissues, after 6 days of IVC, the mean number of intact follicles per 0.1mm^2 tissue are did not change much ($p>0.05$).

The number of intact secondary follicles per 0.1mm^2 in tissues after vitrification-thawing, doesn't differ from fresh ovarian tissues ($p>0.05$). In both, fresh and vitrified tissues, the number of intact secondary follicles after 6 days of IVC increased, the follicles appear morphologically normal, the oocyte is attached to the surrounding granulosa cells, it is however statistically important only in vitrified groups ($p<0.05$).

In general, primordial follicles had a better survival rate during the entire procedure. There is an increase in mean number intact primordial follicles in 0.1mm^2 area in fresh tissues after 6 days of IVC, which is almost statistically important ($p=0.05$), but in vitrified-thawed tissues, more follicles appear deformed or detached from their surrounding granulosa cells after IVC, falling from around 5 to around 2 primordial follicles per 0.1 mm^2, which is not statistically important ($p>0.05$) (Figure 1).

Noticeable loss of intact secondary follicles was seen compared to intact primordial follicles in vitrified tissues without IVC ($p<0.01$), which indicates poor survival of growing follicles after vitrification-thawing, however after 6 days of IVC the difference decreased ($p>0.05$), meaning culturing induced the growth of new morphologically normal follicles. In fresh tissues, there is no significant difference between intact primary and secondary follicles in non-IVC and 6 days of IVC

tissues (p>0.05). Overall, out of all primordial follicles counted, 52.5% of them were morphologically normal and out of all secondary follicles 33.3% were normal (p<0.05).

Table 1. The numbers and proportions of morphologically intact follicles in different treatment groups.

	primordial/primary	secondary	total
Fresh			
noncultured	14/37 (37.8%)	2/6 (33.3%)	16/42 (38.1%)
IVC 6	30/50 (60%)	11/23 (47.8%	41/73 (56.2%)
Vitrified			
noncultured	52/94 (55.3%)	2/15 (13.3%)	54/109 (49.5%)
IVC 6	9/19 (47.4%)	6/19 (31.6%)	15/38 (39.5%)

Proportions indicate normal intact follicles over total counted. Ovarian pieces were divided as follows – fresh, without vitrification and without in vitro culture (noncultured) or without vitrification and with 6 days of in vitro culture (IVC 6), vitrified, those which underwent vitrification procedure and were not cultured or after vitrification were in vitro cultured for 6 days (IVC 6).

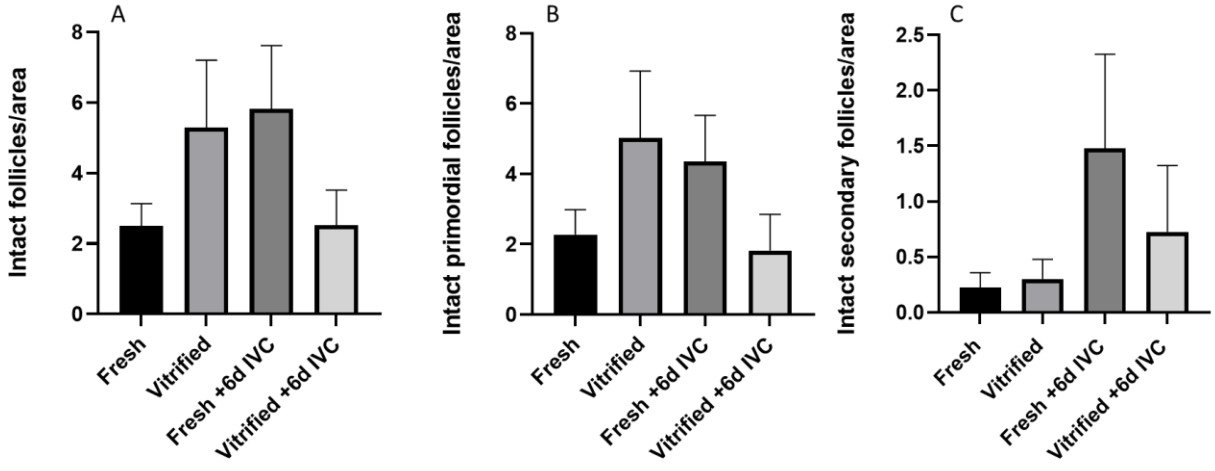

Figure 1. Mean numbers of morphologically intact follicles per area of 0.1 mm^2 (with Standard Error of Mean - SEM) in fresh and vitrified, non-cultured and 6 days in vitro cultured tissues. (A) Comparison of overall morphologically intact follicles among fresh and vitrified, pre- and post- in vitro culture for 6 days (+6d IVC). (B) Comparison of morphologically intact primordial follicles among fresh and vitrified, pre- and post- in vitro culture for 6 days (+6d IVC). (C) Comparison of morphologically intact secondary follicles among fresh and vitrified, pre- and post- in vitro culture for 6 days (+6d IVC).

No significant differences were observed in the stroma of ovarian tissue between fresh and vitrified pieces. Some vitrified tissue pieces showed an increase in areas, where vacuolization was present. Cell density, however, increased in both fresh and vitrified tissues after IVC, less vacuoles were seen in tissues after culture.

2.3.2. Cell proliferation detection using Ki-67

The Ki-67 signal positive cell density in the fresh group was an average of 36 cells/1730 μm^2 and in the vitrified-thawed group 37 cells/1730 μm^2. The density of proliferating cells was similar between fresh and vitrified noncultured tissues. In contrast, after subsequent 6-day in vitro culture (IVC) the difference of density of proliferating cells between fresh and vitrified tissues increased ($p<0.05$), unvitrified tissues with 6 days IVC had an average of 55 cells/1730 μm^2 while vitrified tissues, with 6 days IVC, remained at about 37 cells/1730 μm^2. The increase in proliferating cell density in fresh tissues before and after 6 days of IVC is considered important ($p<0.05$). The average count of proliferating cells per area of 1730 μm^2 surface area in each group is presented in figure 2 and comparison of Ki-67 positive and negative tissues is shown in figure 3.

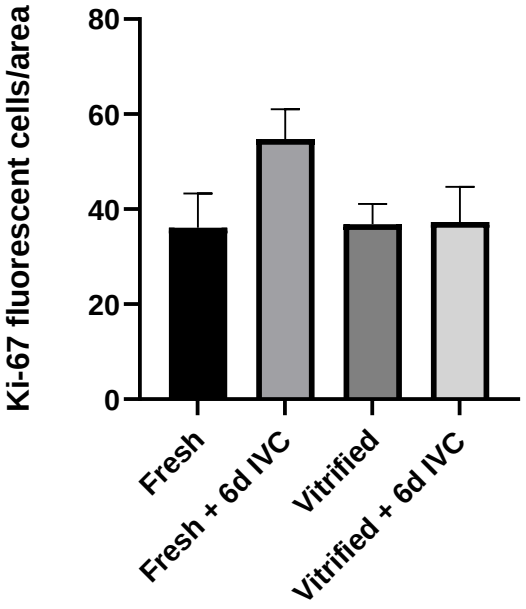

Figure 2. The mean amount (with SEM) of Ki-67 positive cells per 1730 μm^2 area in fresh and vitrified ovarian tissues before and after 6 days culture (6d IVC). The y-axis shows the number

of proliferating cells in a surface area of 1730 µm^2, columns show different average densities of proliferating cells in different groups.

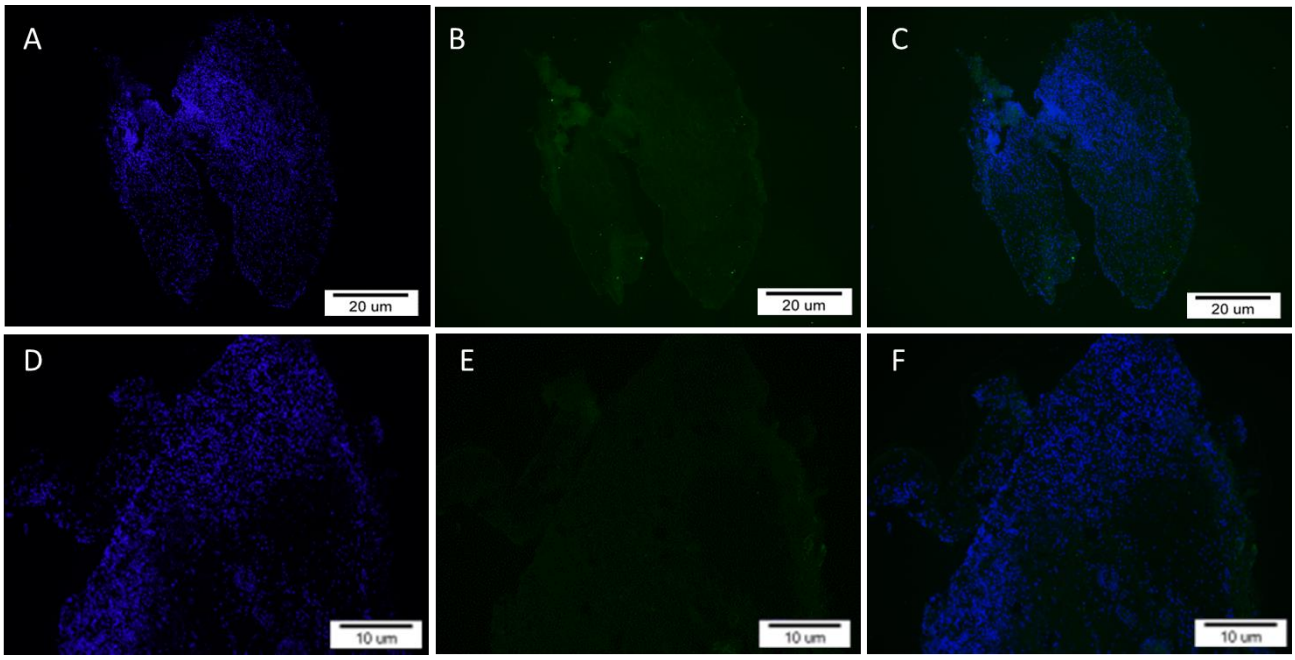

Figure 3. Illustrative images of Ki-67 fluorescence in ovarian tissues. (A–C) Ki-67 positive expression and (D–F) Ki-67 negative expression. (A and D) DAPI, (B and E) Ki-67, and (C and F) merged images; top row (A-C) scale bar = 20 µm, bottom row (D-F) scale bar = 10 µm.

2.3.3. Apoptosis detection using TUNEL

For comparing non cultured fresh tissues with vitrified tissues, TUNEL positive cells were counted, and tissue areas measured. The average TUNEL positive cell count per 2000 µm^2 was significantly different between fresh and vitrified tissues ($p<0.05$), there were more apoptotic cells in cryopreserved tissues. The average TUNEL positive cell count per tissue in fresh ovaries did not exceed 20 cells/2000 µm^2, while in vitrified tissues the cell count was 40 cells per area on average. Apoptosis in all tissues after IVC was spread throughout the entire tissues (figure 4). Though apoptosis was visible throughout all the entire tissues after culture, it remained similar between vitrified and fresh tissues, averaging at about 2000 TUNEL positive cells per area, which comparing to uncultured tissues, is a very significant increase (figure 5).

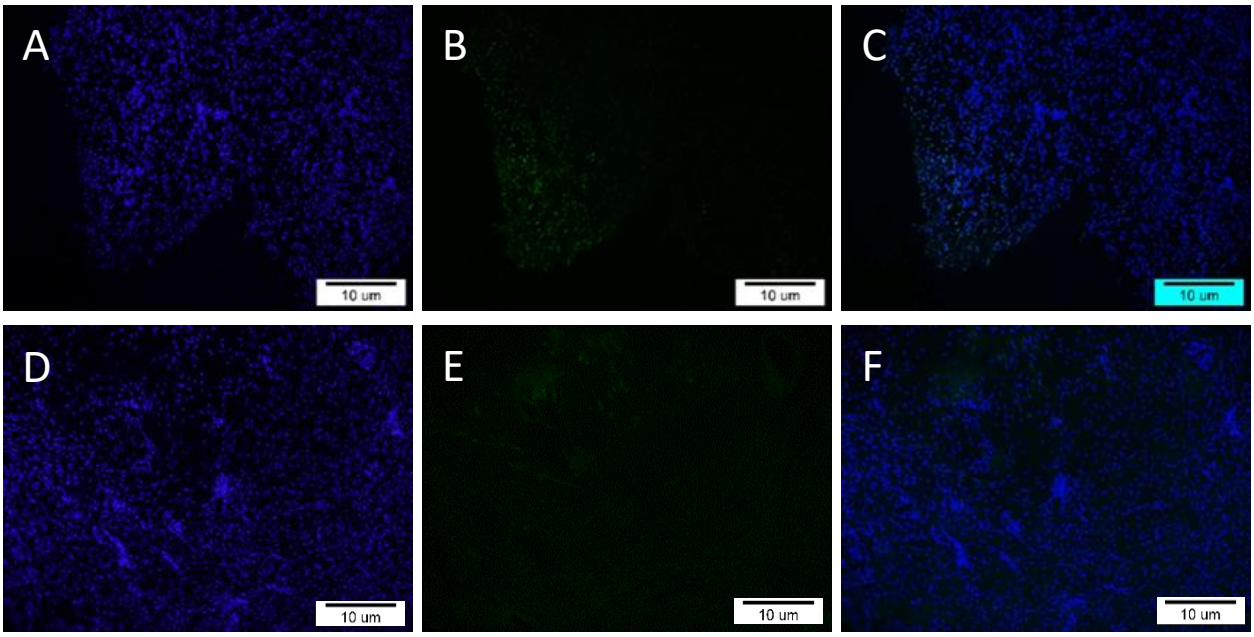

Figure 4. Illustrative images of TUNEL fluorescence in ovarian tissues. (A–C) TUNEL positive and (D–F) negative tissues. (A and D) DAPI, (B and E) TUNEL, and (C and F) merged images; scale bar = 10 µm.

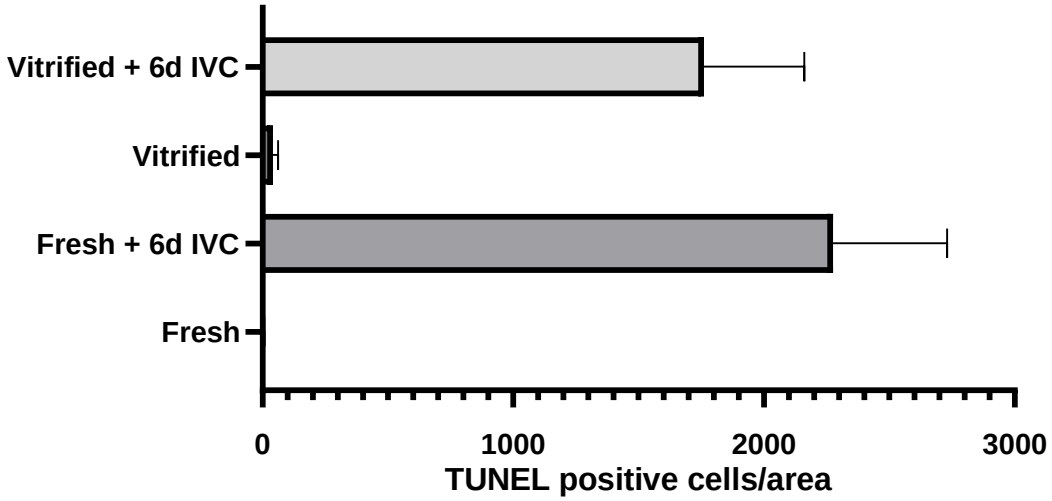

Figure 5. The mean amount (with SEM) of TUNEL positive cells per 2000 µm^2 area in fresh and vitrified ovarian tissues before and after 6 days culture (6d IVC). The y-axis shows the number of apoptotic cells in a surface area of 2000 µm^2, columns show different average densities of apoptotic cells in different groups. Mean number of TUNEL positive cells in Fresh tissues is so small in comparison, it is not visualized on the graph.

2.3.4. Discussion

The aim of this study was to identify what effects does the cryopreservation protocol have on the ovarian cortex of mammals. The vitrification solution had ethylene glycol, ficoll PM70 and sucrose as the main cryoprotectants, similar solutions were used in studies on mice and humans, which had good results regarding apoptosis and tissue integrity (Abdollahi *et al*., 2013; Salehnia *et al*., 2002). However, those studies analyze their result without including in vitro culturing of the tissues, when immediately after thawing the effect of vitrification on the cells may not be yet detectable.

Current results show that overall, the whole procedure, from dissecting the tissue to thawing and in vitro culturing, does damage the tissue as a whole – the amount of intact follicles is relatively low, even in fresh tissues, and apoptosis is high in both fresh and vitrified tissues after culture. There is a significant difference between the number of normal primordial and growing follicles, the latter being negatively affected by the freezing procedure. Secondary follicle count does rise after IVC in vitrified groups, as was shown in a study where much of the damage was mitigated during culture, with the addition of insulin (Mouttham *et al*., 2015). Therefore, adding supplements that support growing follicles could provide better outcomes. Primordial follicles seem to survive vitrification-thawing process quite well, the proportions of intact primordial follicles is greater than growing ones, as have multiple studies shown. However, after culture, results indicate that the vitrification procedure causes the loss of intact primordial follicles in the longer term. A problematic aspect of grafting and freezing the ovarian tissue, that should be paid more attention to, is follicle loss following transplantation due to ischemia and hypoxia (Gavish *et al*., 2014). Current results do not show clearly weather active follicle recruitment is present, so to know if this protocol has the issue with follicle burn-out, analysis of hormone production would be necessary. Results of intact follicles indicate that fresh tissues respond better to IVC, than vitrified tissues, mitigating the damage after dissection. Same can be said looking at proliferation assessment, fresh tissues showed significant increase in proliferation after culture, while vitrified tissues, though not much different from fresh groups, did not display a more active proliferation after IVC. The results so far come in contrast to apoptosis evaluation. Between fresh and vitrified tissues without IVC, there is an increase in apoptotic cells after vitrification, which is inconsistent

with previous studies (Abdollahi *et al*., 2013). After 6 days of IVC both fresh and vitrified cortex pieces appear apoptotic throughout the entire tissue, covered on average in 50% TUNEL positive cells, though there are studies with similar results showing how with the duration of IVC, the intensity of DNA fragmentation increases. In fresh tissues, this could be explained by the stress caused by lengthy procedure of dissection of the ovary. Apoptosis in vitrified cells could be caused by the high concentrations of cryoprotectants (CPA) necessary for vitrification. CPAs have shown to be a cause of accumulation of oxygen radicals, which in high concentration causes oxidative damage and apoptosis (Rahimi et al., 2003). The balance between the tissue piece size and CPA concentrations is very important, as CPAs may not properly penetrate a tissue piece that is too large, while at the same time being too toxic if exposure time is too long for a smaller piece (Kagawa et al., 2009).

To better understand the cause of current results, further test should be done. The vitrification procedure requires high concentrations of cryoprotectants which can be toxic to the tissues, so an extended time of thawing may be required (Sugishita et al., 2022). Current results show contradictory results between intact follicles with proliferation and apoptosis immunostaining, however activity within the tissue could be better understood by measuring gene expressions of both apoptosis and proliferation, as have many studies done (Labrune *et al*., 2020; Abdollahi et al., 2013).

Summary

Cryopreservation of ovarian tissue is an actively developing method of fertility preservation for women going through cancer treatments with the risk of becoming infertile. There are other clinically established methods, like embryo and oocyte cryopreservation, however there are some aspects that puts those two methods at a disatvantage – neither of them can be implemented on pre-pubertal girls, patients with hormone responsive cancers and patients who absolutely cannot delay their cancer treatment for a time needed for ovarian stimulation. At first, ovarian tissue cryopreservation was done by slow freezing, however more recently the method of vitrification was shown to be superior by multiple criterias.

The aim of this thesis was to identify how does vitrification protocol affect the mammalian ovarian cortex, the survival and morphology of follicles, also if proliferation and apoptosis of the cells present in the ovarian cortex are affected. The histological analysis results showed no significant difference in the amount of morphologically normal follicles between fresh and vitrified tissue pieces, mostly the growing follicles were affected. 6 days of subsequent in vitro culture showed that fresh tissues grow somewhat better, while vitrified showed loss of intact follicular density. Ki-67 staining for proliferation showed no difference in proliferating cell density between fresh and vitrified tissues. 6 days of culture did not bring about any changes in vitrified tissues, proliferation did not increase as it did in fresh tissues. TUNEL staining results showed higher apoptosis frequency in vitrified tissues and even more apoptotic cells were found in all tissue pieces that were in 6 days IVC.

Külmsäilitamise mõju imetajate munasarja koorele

Dana-Karolin Rand

Resümee

Üks sagedasemaid riske, mida vähihaigetel naistel pärast keemia- või kiiritusravi täheldatakse, on munasarjade folliikulite ammendumine, stromaalne fibroos ja veresoonte vigastus, mis põhjustab varajase menopausi ja primaarse munasarjade puudulikkuse.

Siiani on naiste viljakuse säilitamiseks kasutatud kolme erinevat meetodit. Küpsete munarakkude või embrüote pikaajaline krüosäilitamine ja munasarjakoe külmsäilitamine. Juba päris ammu on kasutatud embrüo külmsäilitamist, mille efektiivsus on võrreldav värske embrüo siirdamisega see meetod nõuab aga meessoost partnerit. Sel põhjusel hakati kasutama küpsete munarakkude külmsäilitamist. Munarakkude ja embrüo külmsäilitamise üks peamisi probleeme on munasarjade kontrollitud stimulatsiooni nõue. See on ohtlik patsientidele, kellel on hormoonidele reageerivad vähivormid, samuti nõuab stimulatsioon aega, mis on limiteeriv tegur patsientidele, kes peavad koheselt alustama raviplaaniga. Sel põhjusel saab nende patsientide puhul kasutada munasarjakoe külmsäilitamine. Viiakse läbi lihtne biopsia, mida saab rakendada kohe ja ilma hormonaalset stimulatsiooni kasutamata. See on ka peamine lähenemine, mida saab kasutada puberteediealiste tüdrukute puhul.

Selle lõputöö eesmärk oli välja selgitada, kuidas klaasistusprotokoll mõjutab imetajate munasarjakoore tööd.

Munasarja kudede külmsäilitamine hõlmas veiste munasarjade tükeldamist ja tükkide jagamist 4 rühma, millest esimene oli värske, klaasistamata ja kultuuris kasvatamata. Teise rühma moodustasid värsked koed, mis olid kultuuris kasvatatud 6 päeva. Klaasistamiseks inkubeeriti munasarja tükke järk-järgult krüoprotektiivses lahuses ja sukeldati kiiresti vedelasse lämmastikku, kus neid hoiti 6 päeva. Sulatamiseks inkubeeriti klaasistunud koetükke soojendavates lahustes. Sulatamisjärgseid kudesid oli jagatud 2 rühma, millest üks oli klaasistatud-sulatatud ja teine klaasistatud-sulatatud ning kasvatatud kultuuris 6 päeva. Nelja

rühma võrreldi histoloogia ja immunofluorestseeruvate markerite, rakujagunemist ja apoptoosi, abil.

Histoloogilise analüüsi tulemused ei näidanud olulist erinevust värskete ja klaasistunud koetükkide vahel tervete folliikulite suhtes, samuti värskete ja klaasistunud kudede vahel pärast 6 päevast kultuuris kasvatamist. Kui vaadelda värskete ja klaasistatud rühmade tulemusi eraldi, siis värsked, mitteklaasitud koed reageerivad paremini järgnevale kultuuris kasvatamisele, tervete folliikulite hulk suurenes. Sarnased tulemused ilmnesid ki-67 värvimisel rakujagunemise uurimiseks, kus kasvatatud ja kasvatamata värskete ja klaasistatud kudede vahel ei täheldatud märkimisväärset erinevust, samas värsked munasarja tükid jagunesid palju aktiivsemalt. TUNELi värvimise tulemused näitasid klaasistunud kudedes kõrgemat apoptoosi sagedust ja veelgi rohkem apoptootilisi rakke leiti kõigis koetükkides, mis olid 6 päeva kultuuris kasvatatud.

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