FACULTY OF VETERINARY MEDICINE
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# OPTIMALIZATION OF *IN VITRO* BOVINE PRE-ANTRAL FOLLICLE RETRIEVAL BY THE USE OF A PERCOLL® GRADIENT

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## PREAMBLE

In March 2020, when the Belgian government entered the federal phase of crisis management, the research conducted for this thesis was basically completed. Without the pandemic, we probably would have changed a few details in the protocol to improve the results. Nevertheless, the main research could be completed, and the crisis did not affect the outcome of this research significantly. As the communication prior to the arrival of SARS-CoV-2 was mainly electronical anyway, the quality of the communication did not suffer and was kept at the same high level.

# PREFACE

In dit voorwoord wil ik graag een aantal mensen bedanken die hebben bijgedragen tot het ontstaan van deze masterproef.

Allereerst wil ik mijn promotor Prof. dr. Peter Bols bedanken. Op het weg naar het tot stand komen van deze titel en uiteindelijk deze masterproef hebben we heel wat gesprekken gevoerd en samen overlegd in welke richting een onderzoek in uw vakgroep zou kunnen gaan. Het was een goed gevoel om eens na al deze jaren 'theorie van buiten blokken' eens mijn eigen hoofd te mogen gebruiken en eens buiten de wereld van de practici te kijken. Hierdoor heb ik heel veel bijgeleerd – binnen en buiten de diergeneeskunde. Hartelijk bedankt voor uw inzet en het vertrouwen in mijn vermogen deze uitdaging aan te gaan. Ook veel dank aan Prof. dr. Wim Van Den Broeck om mij als co-promotor te begeleiden.

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Mijn lieve vrienden, mijn lotgenoten. We hebben samen zo veel beleefd, zo veel samen meegemaakt. De afgelopen 6 jaar zal ik nooit vergeten. Bedankt voor alles!

Und zum Schluss ein dankbares, unendlich großes und nicht in Worte zu fassendes DANKE an meine Familie und Freunde. Ihr habt immer an mich geglaubt, mich unterstützt in meinem so unerreichbar scheinendem Traum, Tierärztin zu werden. Alle Höhen und Tiefen habt ihr mit mir durchlebt. Ohne euch hätte ich es nie geschafft. Danke, dass es euch gibt.

*"The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day."*

- *Albert Einstein* -

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# 1) LIST OF ABBREVATIONS

| | |
|---|---|
| AI | Artificial Insemination |
| AMH | Anti-Müllerian Hormone |
| ART | Artificial Reproductive Technique |
| Cx | Connexin |
| CCPE | Cumulus cell process ending |
| COC | Cumulus oocyte complex |
| DDR | DNA Damage Response |
| DGC | Density Gradient Centrifugation |
| FP | Fertility Preservation |
| FSH | Follicle Stimulating Hormone |
| GC | Granulosa Cell |
| GDF-9 | Growth and Differentiation Factor-9 |
| GJ | Gap Junction |
| GJC | Gap Junction Channel |
| GJIC | Gap Junctional Intercellular Communication |
| ICSI | Intracytoplasmatic Sperm Injection |
| IVF | *In Vitro* Fertilization |
| IVM | *In Vitro* Maturation |
| MOET | Multiple Ovulation and Embryo Transfer |
| OTC | Ovarian Tissue Cryopreservation |
| OTT | Ovarian Tissue Transplantation |
| PAF | Preantral Follicle |
| PGC | Primordial Germ Cell |
| PI3K | Phosphatidylinositol 3-Kinase |
| PKB | Protein Kinase B |
| PMF | Primordial Follicle |
| PTEN | Phosphatase and tensin homologue of chromosome 10 |
| ROS | Reactive Oxygen Species |
| SLC | Single Layer Centrifugation |
| TGF-β | Transforming Growth Factor-β |
| VEGF | Vascular Endothelial Growth Factor |

## 2) ABSTRACT

**Objective:** The increasing advances in scientific and biotechnical technologies as well as enhanced knowledge around the causes of infertility resulted in the growth of research in the reproductive field of humans and animals. In females, the isolation and preservation of preantral follicles has gained more and more attention as they form a large and resilient pool of genetic material that can be processed in various ways. Concerning the possible methods, this can lead to a 'problem of choice'. To partly address this issue, this thesis focusses on the possibilities of fertility preservation strategies in males and females as well as the advantages and challenges that are associated with the approaches. Furthermore, the research conducted in this thesis uses a bovine *in vitro* model to attempt the development of an improved isolation protocol for the retrieval of a maximum number of good quality bovine preantral follicles by the use of a Percoll® gradient.

**Material and methods:** Follicles where isolated from the cortex mechanically using a grit cutting pattern and a tissue disperser. The follicles were recovered by applying a discontinuous Percoll® density gradient method. Follicular viability was assessed by a Neutral Red Stain. Stained and non-stained follicles were counted and scaled into four categories.

**Result(s):** Out of a total of 2,360 retrieved follicles, 53% (n = 1255) were found at the 1.09 g/ml Percoll® layer and 41% (n = 972) were located at the 1.06 g/ml Percoll® layer, which represents 94% of the entire retrieved follicles. In total, 73% of the follicles were positively stained by Neutral Red and were therefore classified as viable.

**Conclusion(s):** The Percoll® density gradient method allows the retrieval of a large quantity of viable follicles as long as the manipulation time is kept short.

**Key words:** fertility preservation, isolation, ovary, preantral follicle, retrieval, bovine, Percoll density gradient

# 3) SAMENVATTING

**Doelstelling:** Naast een toenemende vooruitgang in wetenschappelijke en biotechnische technologieën heeft ook een verbeterde kennis van de oorzaken van onvruchtbaarheid ertoe geleid dat het onderzoek in het kader van de voortplanting van mens en dier sterk is toegenomen. De focus wordt steeds meer gericht op de isolatie en conservatie van preantrale follikels. Deze vormen namelijk een grote en resistente pool van genetisch materiaal dat op verschillende manieren verder verwerkt kan worden. Dit kan leiden tot een keuzeprobleem. Om deze uitdaging toe te lichten gaat zich deze thesis focusseren op de verschillende opties voor de conservatie van fertiliteit in mannen en vrouwen als ook de voor- en nadelen die hiermee gepaard gaan. Het onderzoek dat uitgevoerd werd maakt gebruik van een bovien *in vitro* model. Hiermee wordt getracht om een verbeterd isolatie protocol voor de winning van preantrale follikels van goede kwaliteit te ontwikkelen met behulp van een Percoll® densiteit gradiënt.

**Materiaal en methode:** De follikels werden mechanisch van de cortex geïsoleerd met behulp van het insnijden van een raster en het dispergeren van het weefsel met een gespecialiseerde weefsel mixer. De follikels werden gewonnen met behulp van een discontinue Percoll® densiteit gradiënt methode. De levensvatbaarheid van de follikels werd geëvalueerd met een Neutraal Rood kleuring. Gekleurde en niet-gekleurde follikels werden geteld en verdeeld in vier categorieën.

**Resultaat:** Van de 2,360 gewonnen follikels, 53% (n = 1255) warden gevonden in de 1.09 g/ml Percoll® lag en 41% (n = 972) werden gelokaliseerd in de 1.06 g/ml Percoll® laag. Dit representeert 94% van het gehele aantal gewonnen follikels. In totaal waren 73% van de follikels positief gekleurd met neutraal rood en daarom gecategoriseerd als levensvatbaar.

**Conclusie:** De Percoll® densiteit gradiënt methode laat de winning van een groot aantal levensvatbare follikels toe zolang de periode van manipulatie kort wordt gehouden.

# 4) INTRODUCTION

In the past years, cancer incidence rates increased globally (Moukayed and Grant, 2017). According to recent data published by the American Chemical Society Journals, the cumulative risk of incidence implies that 1 in 8 men and 1 in 10 women will develop cancer during their lifetime (Bray et al., 2018). Before reaching the age of 40, approximately 2% of all women will have been diagnosed with an invasive cancer. Due to a persisting tendency to delay pregnancies, many women will not yet have started or achieved their family forming goal. As cancer treatments are constantly improving, fortunately the survival rates have increased. Nevertheless, population-based studies indicated that the prospect of having a live birth is reduced by 30-50% in cancer survivors in comparison to controls (Bray et al., 2018). The probable infertility is mainly caused by damage to the ovarian tissue as a result of therapeutic procedures such as radiation and chemotherapy (Chung et al., 2013). Looking at survivors of childhood cancer, Green et al. (2009) stated that the prospect of ever getting pregnant is reduced by 19%, provided the patients haven't faced surgical sterility already. In addition to the diagnosis of cancer, the possibility of a treatment-induced infertility is a substantial source of distress in patients (Chung et al., 2013). The will to survive on one side, facing the desire to eventually form a family with biological offspring on the other side, has sparked the interest in fertility preservation strategies in women. Additionally, the necessity of preservation of genetic material has also increased for endangered animal species as well as in animals with remarkable genetic attributes (Bus et al., 2019). The most preferential approach for conserving genetic variation has been preserving and securing a large number of habitats. In a growing modern world with increasing numbers of people in need of resources, this strategy is not sufficient to provide enough wild space to guarantee self-sufficient and healthy populations of all species (Comizzoli et al., 2010).

Nearly 5% of the births in developed countries have been made possible through the use of assisted reproductive techniques (ARTs) (Beydola et al., 2013). ARTs can be defined as the methods used to achieve pregnancy by artificial or partially artificial procedures, such as ovulation induction, intrauterine insemination, oocyte retrieval and *in vitro* embryo production in humans (Langbeen et al., 2015a). Despite an increasing demand for ART treatment in Europe, conclusions on potential epigenetic effects and perinatal problems following ART are still inconclusive but are being researched intensively. This also applies to the use of ARTs in livestock, where artificial insemination, multiple ovulation and embryo transfer programs (MOET) and specifically cloning remain important research topics. Nearly all of these technologies' intent to increase the speed of genetic selection of valuable (production) characteristics. While the main target of ARTs is the immediate reproductive success fulfilled by the achievement of a pregnancy, fertility preservation (FP) aims to conserve and restore fertility while adjourning reproductive activity (Langbeen et al., 2015a). Regarding FP in the male, the focus mainly lies on cryopreservation of (epidydimal) sperm in human and veterinary medicine. In humans, sperm is banked ahead of treating oncological and autoimmune diseases featuring gonadotoxic products. On the contrary, sperm from breeding males displaying profiTable characteristics is selected and preserved for artificial insemination in animals.

One of the main limiting factors of male FP is the need for mature sperm which can only be acquired of adult and not prepubertal donors (Ehmcke and Schlatt, 2008). Nevertheless, the use of completely immotile sperm in intracytoplasmatic sperm injection (ICSI) has resulted in successful clinical outcomes and healthy offspring (Chen et al., 2017). In human FP, ICIS offers reproductive opportunities to problematic patients as it only requires live but not motile sperm (Nordhoff et al., 2013). The use of

either frozen-thawed motile sperm, percutaneous epididymal sperm aspiration (PESA) or testicular aspiration (TESA) in ICSI showed similar results in many studies (Chen et al., 2017). However, only motile sperm are commonly selected for cryopreservation, which results in a missed FP opportunity for patients with immotile sperm (Chen et al., 2017). Since fertilization of an oocyte is the ultimate goal of male FP, sperm quality plays a major role. Therefor *in vitro* sperm preparation and selection techniques attempt to imitate the natural process in which viable sperm is separated from other fractions of the ejaculate due to active migration through cervical mucus (Beydola et al., 2013).

Whereas the techniques for the retrieval and quality management of spermatozoa are broadly elaborated, FP techniques for women and other female mammals are more complex and not yet fully explored. Most mammalian species are known to have a limited resting pool of immature follicles available within the ovaries. Preantral follicles (PAFs) form the most important part of that pool and are often described as the *'ovarian reserve'* as they are preserving the vast majority of female reproductive capacity, supplying oocytes at regular intervals throughout reproductive life. In the so-called *'preantral phase'*, certain follicles undergo activation, are *'recruited'* and start growing to ultimately enter *'selection'* and become antral and *'dominant'* after which finally ovulation can take place (see below, Langbeen *et al*., 2015a; Sjaastad *et al*., 2016). However, about 99,9% of the early PAFs will undergo atresia resulting in only a small number of oocytes developing to the ovulatory stage (Oktem and Urman, 2010). The estimated duration of development from the primordial to the ovulatory follicle stage differs considerably among species, taking for example 180 days in cattle and sheep, which is comparable to the required period in humans (205 days). In mice, the development will only take 20 days which differs substantially from the other 3 species (Langbeen et al., 2015a). Next to the above-mentioned destruction of PAFs due to ootoxic cancer treatments, a depletion of the ovarian reserve in humans occurs due to physiological processes such as menopause (Li et al., 2012) or due to pathological reasons such as premature ovarian failure (Roness et al., 2013). In those cases, FP can lead to a solution to ensure reproductive capacity.

Modern FP techniques for women include ovarian transposition, ovarian tissue cryopreservation (OTC) containing PAFs, as well as the cryopreservation of both embryos and unfertilized oocytes. While cryopreservation of embryos produced *in vivo* and *in vitro* has become a common technique in animals, freezing of ovarian tissue is still in its experimental phase, both in humans and animals (Niemann and Wrencycki, 2018). In female cancer patients, cryopreservation of either embryos or oocytes requires the patient to be of pubertal age, have a partner or use the sperm of a donor. Furthermore, the patient needs possess the ability to undergo a cycle of ovarian stimulation, which results in an undesired delay of the onset of cancer treatment (Donnez et al, 2006). Under these circumstances, OTC is the only possible option. On the downside, this technique is not recommended for patients with types of cancer with a medium to high risk of metastasis in ovarian tissue, such as leukemia. The risk of re-introducing malignant cells at the moment of auto-transplantation of cryopreserved ovarian tissue is simply too high (Rosendahl et al., 2013). Isolation and cryopreservation of PAFs (before or following tissue cryopreservation) from ovarian tissue could be a more reliable alternative to restore reproductive capacity in those patients. The PAFs would be isolated from ovarian tissue for *in vitro* growth, maturation and fertilization. Moreover, frozen-thawed isolated PAFs could be auto-transplanted in residual ovarian tissue *in situ* (Fisch and Abir, 2018).

In animals, (fertility) preservation strategies are frequently complicated due to the time-sensitive character of the procedure. The retrieval and cryopreservation of ovarian tissue often is the only possibility for conservation in case of an unexpected death or killing of an animal from an endangered species. If the animal has been dead for multiple hours, the isolation of PAFs from the surrounding tissue might be useful as it avoids the cryopreservation of ovarian tissue samples that do not contain follicles (Bus et al., 2019). As mentioned above, the main limiting factor is the lack of routinely available *in vitro* fertilization (IVF) and maturation (IVM) technologies for numerous species. For the preservation of a donor's reproductive potential, ovarian tissue xenotransplantation can be an option, though it is still an experimental approach. As investigated in rodent (Snow et al., 2002) and wombat (Mattiske et al, 2002) trials, xenografting is a promising technique for the production of mature oocytes from endangered species for the use in ARTs. Moreover, nuclear transfer for the preservation of critically endangered species can be done through the use of mature oocytes from non-endangered species (Bus et al., 2019). Finally, according to Amorim and Shikanov (2016), the creation of an *in vitro* 'artificial ovary' is one of the most recent research paths. Isolated PAFs can be encapsulated in a biological degradable matrix and transplanted into residual ovarian tissue or another location in the body. The matrix that will not only encapsulate and protect isolated follicles but will also safeguard autologous ovarian cells and bioactive factors that are fundamental for the survival and development of the follicle.

The cryopreservation of PAFs specifically plays a major role in ovarian tissue cryobanking and the creation of alternative storage techniques for isolated follicles. Due to the limited access to human gametes out of ethical restrictions, this study was based on a bovine *in vitro* model. This model is commonly used because of its similarities with the human ovary including size and structure (Kagawa et al., 2009) as well as ovarian function and oocyte characteristics (Campbell et al., 2003). Regarding the unlimited access to bovine ovaries from slaughterhouses, this model is an ideal option for human reproductive research. For a review on the opportunities using the bovine model, please consult Langbeen et al. (2015b). Even though approaches and techniques have been investigated broadly, there still are limitations and a lack of knowledge in the many subfields of fertility preservation. The isolation of PAFs is one of those branches. Various approaches have been studied to ensure isolation that results in the greatest yields of follicles, preferably of high quality. Although different strategies are available, there is no 'golden standard protocol' available yet, which displays that there is a need for optimalization. However, multiple studies on FP have peeked into FP strategies of the other gender, trying to find the solution in their protocols. That is why an insight in both female and male FP can help to improve protocols through the coalescence of their approaches.

# 5) LITERATURE REVIEW

## 5.1) THE OVARY

The ovaries or female gonads represent one important subpart of the female internal reproductive organs. One of their two closely integrated functions is the storage and cyclic release of the female germ cells. Furthermore, the ovaries are the production sites of female sex hormones. Not only the development of the reproductive organs is modulated by these hormones but also the initiation of sexual behavior. During pregnancy they also assist in the adjustment of the maternal body to the requirements of the growing fetus. In most species the ovaries are located in the abdominal cavity just caudal of the kidneys. Their shape ranges from round to ovoid. In animals with a seasonal estrus they show a variation in size depending on their current state of function. The broad uterine ligament (*Lig. latum uteri)* secures the ovaries cranially whereas the *ligamentum ovarii proprium* ensures its attachment to the uterus. The surface of the ovary is composed of an epithelial monolayer. Beneath this modified serosa, the *tunica albuginea* is located. The underlying cortex is mainly composed of connective tissue that is well supplied with blood vessels (Gille, 2008). The cortex contains numerous oocytes which are embedded in follicles at different developmental stages, ranging from primordial to preovulatory (Fig. 1). The differentiation between the stages of preantral and antral follicles is contingent upon the presence of a central cavity, the antrum, filled with fluid formed by the granulosa cells (GCs) (Sjaastad et al., 2016). The most immature follicle type is the primordial follicle (PMF). It is also the most abundant in the follicular reserve pool (Bus et al., 2019). Woods and Tilly (2013) hypothesize that the follicle pool in mice is partially sustained by a rare population of premeiotic germ cells that are able to create new oocytes postnatally. Contrary to rodents, the number of PMFs in the ovaries is finite in primates and most domesticated animal species thus determining the reproductive life span in females (Eppig, 2001; Fortune, 2003). With 90% of the total follicular reserve, PAFs account for the majority of follicles located in the ovarian cortex. Considering their large number and given that PMFs are the upmost starting point for *in vitro* culture, PAFs provide a considerable potential source of genetic material (Araújo et al., 2014).

### *5.1.1) FOLLICLE FORMATION, ACTIVATION AND GROWTH*

During the early organogenesis, primordial germ cells (PGCs) migrate from the yolk sac into the gonadal primordium. After arriving and proliferating in the evolving female gonad, the PGCs are surrounded by flat somatic follicular cells. At this stage, the PGCs are referred to as '*oogonia'*. These oogonia display high mitotic activity before entering meiosis. The formation of a coelomic epithelium around the oogonia turns them into PMFs (Oktem and Urman, 2010). The PMFs are encircled by flat follicular (pre-granulosa) cells that rest on a basement membrane. The latter disconnects them from the surrounding stromal cells. Thus, the follicles contain the oocyte. Oocytes that are not enclosed in a follicle will undergo apoptosis (Hyttel et al., 2010). Eppig (2001) states that the oocyte itself is crucial for the formation of the follicle as it assures follicle assembly. On the contrary, the seminiferous tubule (being the male equivalent) can develop without the presence of spermatocytes.

The primordial follicles constitute the resting oocyte pool that will provide the female during her whole reproductive life span. The pre-granulosa cells will continue to multiply only after the initiation of follicular growth, also referred to as 'primordial follicular activation' (Araújo et al, 2014). The meiotic arrest is necessary for the PMFs to gain the competence of being activated (for review, see Aerts and

Bols, 2010). After activation, the PMFs will leave the resting and enter the growing pool which makes the activation an irreversible step (Araújo et al, 2014). With the activation of the PMF, the pre-GCs will modify into a cuboidal monolayer around the oocyte creating the so-called '*primary follicle*'. Moreover, the cuboidal layer of cells is now specified as GCs. The entering of this phase also initiates oocyte growth. In domestic species, the diameter of the oocyte expands from less than 30 to more than 120 µm. However, the vast majority of follicles that enters the growth phase will not be able to finish it. Most follicles will degenerate through atresia leaving only a minority to develop into a follicle ready for ovulation (Hyttel et al., 2010).

Under the influence of growth and differentiation factors such as vascular endothelial growth factor (VEGF) and growth and differentiation factor-9 (GDF-9), primary follicles will resume in the activated growth phase (Araújo et al., 2014). The GCs proliferate, surrounding the oocyte with various cell layers through which the primary follicle becomes a secondary follicle. Furthermore, the oocyte and GCs generate glycoproteins that assemble in between the two, forming the zona pellucida. The oocyte is nourished by the surrounding GCs through gap junctions that form at the end of the cumulus cell process endings that penetrate the zona pellucida. The stromal cells on the outside of the basal lamina differentiate into thecal cells, with an outer *theca externa* as a supportive connective tissue and an inner *theca interna* as a layer of steroid-producing cells. Thecal cells produce androgens when stimulated by LH and offer them to GCs producing estradiol following FSH stimulation (Hyttel et al., 2010). The following developmental stage, referred to as the early-antral or tertiary follicle, is characterized by the appearance of fluid filled cavities in between the GCs that will eventually coalesce into one antral cavity. In cattle, this type of follicles has been identified around day 220 of gestation (Araújo et al, 2014). While the antrum is expanding, the GCs from a protrusion around the oocyte, the '*cumulus oophorus'* containing granulosa cells that are named 'cumulus cells', protruding into the antrum. Together with the oocyte, they form the cumulus-oocyte-complex or COC (Fair et al., 1997) that sticks to the inner wall of the follicle until the moment of ovulation. Under the influence of increasing plasma levels of follicle stimulating hormone (FSH), recruited follicles start to produce and secrete estradiol. This secretion facilitates the ongoing follicular development. Only a few follicles among the recruited group are selected for further maturation (Oktem and Urman, 2010). Depending on the species, one or more dominant follicles develop into preovulatory or Graafian follicles, awaiting the gonadotrophin signal for ovulation (Sjaastad et al., 2016).

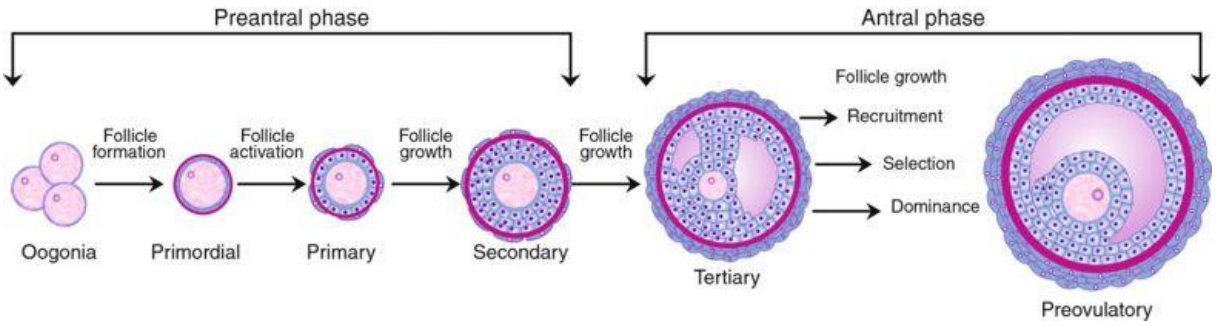


**Figure 1:** Growth of the follicle until the preovulatory stage. In the preantral phase, the oogonium forms a primordial follicle which is activated and thus creates a primary follicle. Continuous growth of the primary follicle leads to the secondary follicle stage is reached. As the secondary follicle keeps on growing, it enters the antral phase. Upon formation of antral cavities, a tertiary follicle is created. After passing through several stages of growth, recruitment, selection and dominance, the preovulatory follicle is ultimately formed (Araújo et al., 2014).

### *5.1.2) PHYSIOLOGICAL AND HISTOLOGICAL ASPECTS OF 'PREANTRAL' FOLLICLES (PAFs)*

As stated above, PAFs account for the majority of follicles located in the ovarian cortex and therefor represent the follicular reserve that determines the length of a woman's reproductive life. Due to their large quantity and considerable source of genetic material (Araújo et al., 2014), PAFs are put at the heart of FP. However, with all the techniques available, there comes a risk of damaging the sensible ultra-structure and survival mechanisms of PAFs. That is why some of these special characteristics of PAFs need to be highlighted and understood, before determining the 'ideal' FP strategy.

Once the resting pool is formed by the PMFs, they can enter one of three possible pathways: (1) staying in the dormant state, (2) dying in their quiescent state, or (3) being activated and entering the growing pool followed by either atresia or continued development (Zhang et al., 2012). These pathways are controlled by both stimulating and inhibiting factors (Oktem and Urman, 2010). Activation, growth and survival of PAFs is dependent on several growth factors, hormones and local factors (Fig. 2). John et al. (2009) stated that the most important signaling pathway for PMF activation is the phosphatidylinositol 3-kinase (PI3K)/phosphatase and tensin homologue of chromosome 10 (PTEN)/protein kinase B (PKB, Akt) pathway. Different studies showed adverse effects on follicular development due to interference with the PI3K pathway (Castrillon et al., 2003; John et al., 2009; Reddy et al., 2010). As Maidarti et al. (2020) recently stated, this can be explained by present evidence that the pathway overlaps with the DNA damage response (DDR) in cells. A strongly increased activity of PI3K/Akt is associated with ovarian ageing and a reduction in the number of PMFs. Furthermore, ovarian ageing is linked to a flawed DDR within oocytes which can be caused by DNA damaging agents, such as chemotherapeutic agents (Maidarti et al., 2020).

Another important factor in the activation of PMFs is anti-Müllerian hormone (AMH), a paracrine factor belonging to the transforming growth factor-β (TGF-β) family that is synthesized by the GCs of growing follicles. The moment follicles start to grow, the production and secretion of AMH increases. The expression suddenly decreases at the moment when follicles are selected for dominance. Several studies have shown that the serum concentration of AMH decreases in association with the approach of menopause in women (Anderson and Wallace, 2013). In addition, other members of the TGF-β family operate as extracellular ligands involved in cell proliferation, differentiation, apoptosis and cell migration (Massague and Wotton, 2000).

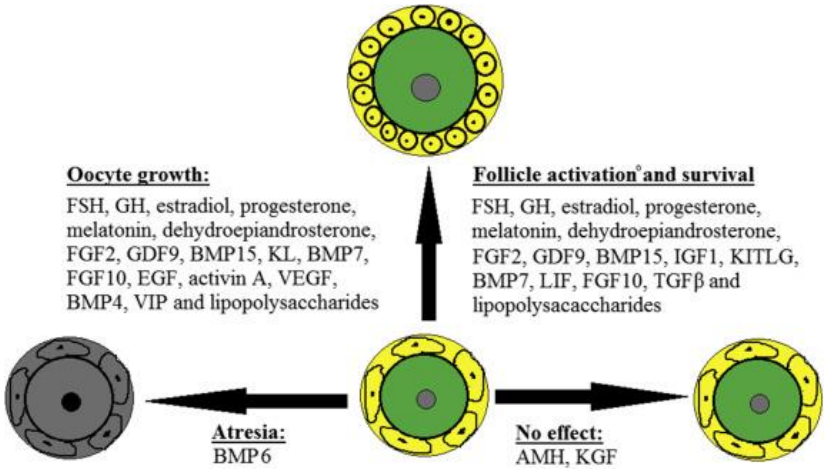


**Figure 2:** Factors controlling the activation, growth and survival of primordial follicles. (Silva et al., 2016)

In addition to paracrine signaling, the interaction between cells plays an important role in regulating cellular processes. A way neighboring cells communicate is through gap junction channels (GJCs), that connects the cytoplasms of bordering cells directly (Su et al., 2014). These intercellular membrane channels ensure electrical and chemical coupling between cells by allowing the exchange of small molecules up to 1 kDa in size (Kidder et al., 2002). A range of secondary messengers, small metabolites and different ions such as potassium can be transferred, which shows that gap junctional intercellular communication (GJIC) is crucial for the establishment and control of intracellular homeostasis. Furthermore, several additional functions such as modulation of signaling pathways and direct protein interactions have been described (Aasen et al., 2018). Different studies have shown that changes in this communicational system can lead to diverse neoplastic, cardiac and developmental diseases in humans (Su et al., 2014). Additionally, GJIC manages signaling and function of numerous other organ systems such as the central nervous system, lens, liver, lung, reproductive system, bone, skin and many more (Kar et al., 2012).

GJIC are formed by connexins, a family of vertebrate proteins. When connexins oligomerize into a hexameric hemichannel (connexon) and connect with another connexon from a neighboring cell, a GJC is formed (Beyer et al., 2009). Each connexin displays a tissue- or cell-type-specific expression and almost every cell type forms more than one connexin isoform (Gershon et al., 2008). Accordingly, the hexameric connexons can be composed of more than one connexin isoform, which means they can be homomeric or heteromeric. The resulting gap junction (GJ) therefore can be homotypic or heterotypic (Evans et al., 2009). This ability of variation in channel formation adds an even greater possibility for functionally modulating GJs. Until now, 21 connexin genes have been identified in the human genome. Although a variety of lower multicellular organisms possesses intercellular channels as well as GJs, genes coding for connexins have only been described in deuterostomes (Beyer et al., 2009). All connexin proteins have a resembling structural organization. They contain four transmembrane, three cytosolic and two extracellular subdomains (Fig. 3). The carboxy-domain, one of the cytosolic subdomains, is most prone to changes – as well in length as in composition. It forms the protein-interaction-site and plays a critical role in the functioning of GJs. The aminoterminal does not vary in between the members of the connexin (Cx) family. It is capable of self-assembly and the formation of hexamers (Kardami et al., 2007).

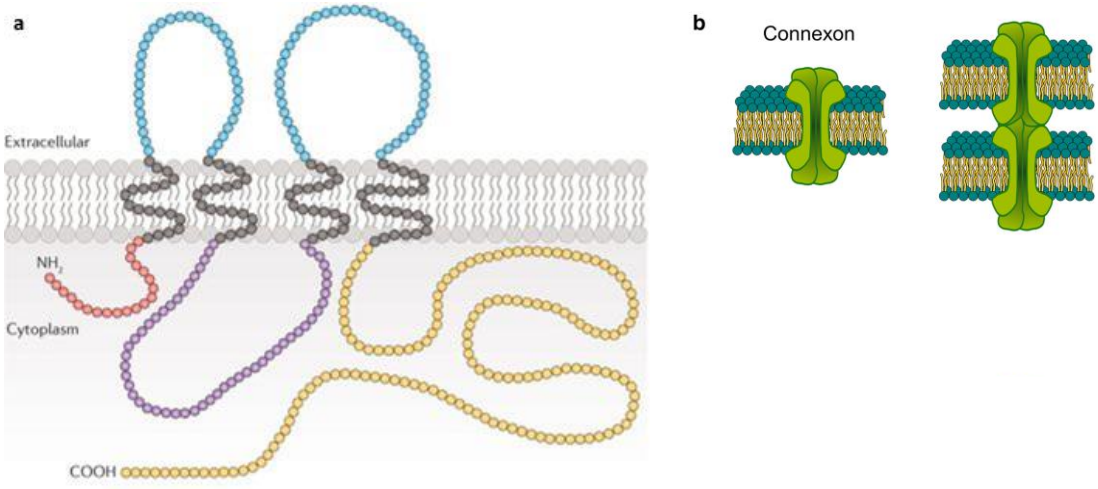


**Figure 3** (**a**) Structural organization of connexins, containing of the three different subdomains of connexins (Laird and Lampe, 2018). (**b**) Morphology of connexons and gap junction intercellular communication (Naus and Giaume, 2016).

Except for erythrocytes, mature sperm cells and differentiated skeletal muscle cells, almost every cell type in vertebrates expresses connexins. However, there is a variation in distribution pattern and quantity of connexins regarding species and cell type. Numerous studies showed that connexins play a pivotal role in reproductive organs. In humans, treating pregnant women with the antimalaria drug mefloquine, increases the risk of induced abortion and stillbirth. According to Winterhager and Kidder (2015), the blockage of GJ function by the active substance is responsible for a loss of intercellular communication in the embryonic state which is later accompanied by a deterioration of placental growth. In cattle, connexins such as Cx43, are for example localized in placental connective tissue (Mansouri-Attia et al., 2009; Bauersachs et al., 2005). In the mare, only Cx43 could be found in the sub-apical plasma membrane of the endometrium whereas none of the previously mentioned connexins could be found in the pig (Day et al., 1998). Pfarrer et al. (2006) detected the expression of Cx26, Cx32 and Cx43 in bovine placentomes during pregnancy. In evolving follicles, the growing oocyte is connected to its neighboring follicle cells by GJs, forming a functional syncytium (Kidder et al., 2002). According to various studies, the main connexins expressed during the development of ovarian follicles are Cx26, Cx37 and Cx43. While the PMF is formed, the oocyte was blocked in its first meiotic arrest. This means that the oocytes are still uncapable to be fertilized and will need to gain their fertilizing capacity throughout their growing process. During the development of the follicles, their diameter almost expands by a factor of 100 (Aerts and Bols, 2010). Whereas the oocyte of the bovine PMF only contains Cx26 (Johnson et al., 1999), the oocyte of the primary follicle also expresses Cx37 in humans (Kibschull et al., 2015) and cattle (Nuttnick et al., 2002). The GCs of the primary follicle will develop Cx43 in pigs (Lenhart et al., 1998; Melton et al., 2001), cattle (Johnson et al., 1999), sheep (Borowczyk et al., 2006a) and humans (Kibschull et al., 2015). At this stage, the GCs of PAFs are connected with the oocyte by an extension that runs through the zona pellucida, the so-called '*cumulus cell process endings'* (CCPEs). The cytoplasm of the extension gets connected to the oocyte by Cx37, while Cx43 mainly provides the link among the GCs (Kidder et al., 2009). The coupling has proven to be necessary to maintain GC proliferation during the follicle development (Borowczyk et al., 2006a). While the expression levels of Cx37 in cattle decrease significantly at the moment of antrum formation, the expression of Cx43 highly increases, even in corpora lutea (Gershon et al., 2008; Nuttnick et al., 2002). Thus, in the stage preceding antrum formation, Cx37 can be found in the oocytes of cattle (Nuttnick et al., 2002) and humans (Kibschull et al., 2015). Cx43 is expressed by human (Kibschull et al., 2015), bovine (Nuttnick et al., 2002; Johnson et al., 1999) and porcine (Melton et al., 2001) GCs.

The follicle will achieve meiotic competence with the formation of an antrum, thus transitioning from a pre-antral to an antral follicle. In humans and cattle, the formation of an antrum is not mandatory for gaining meiotic competence. However, it has been shown that follicular growth will benefit the ability to overcome meiotic arrest (Aerts and Bols, 2010). Cx26 will still be expressed by different cell types in the antral follicle. In pigs and sheep Cx26 can only be detected in theca cells (Borowczyk et al., 2006a), while in cattle it is also found in GCs (Johnson et al., 1999). Ovine granulosa and thecal cells in the antral follicle form Cx37 (Borowczyk et al., 2006b).

The physiological and histological aspects described above show once more the delicate balance between survival and death of follicles that needs to be considered while processing and handling those. These challenges will become more apparent in the following pages as they will give an overview of FP methods in humans and animal models.

## 5.2) OVERVIEW OF FERTILITY PRESERVATION POSSIBILITES IN HUMANS AND ANIMAL MODELS

To preserve fertility in females, numerous strategies have been developed so far, mostly based on the patient's age and the presence of a partner. In the case of cancer, the treatment method and possibility of treatment delay have to be taken into account (Kim et al., 2018). While some of those methods still remain 'theoretical', others have led to promising results (Vanacker et al., 2013). Different methods have been evaluated and tested in the field, including (1) cryopreservation of embryos, (2) cryopreservation of oocytes for future IVF, (3) cryopreservation of ovarian tissue or the whole ovary for prospective transplantation, (4) cryopreservation of ovarian tissue or isolated follicles for *in vitro* growth and maturation, (5) ovarian transposition preceding radiotherapy, (6) hormonal protection using GnRH-analogs, and (7) pharmacological protection with anti-apoptotic agents such as sphingosine-1-phosphate (Kim, 2006). As cryopreservation – either by slow freezing or vitrification – of ovarian tissue is the only method available for prepubertal cancer patients and female cancer patients whose treatment cannot be delayed (Bus et al., 2019), this option will be discussed in more detail. Furthermore, the approaches to follicle isolation, as a 'hot topic' in the current FP research (Santos et al., 2006; Shi et al., 2007, Smitz et al., 2010; Aghadavod et al., 2013; Telfer and Zelinski, 2013; Araújo et al., 2014; Bus et al., 2019; Szymanska et al., 2018), will be extensively illustrated. Even though science has presented several options for FP in females, up until today none has proven to be as trustworthy as sperm banking in males. Moreover, all of the methods in females mentioned above require the use of pharmaceuticals and/or invasive techniques.

Although the main focus of male and female FP strategies lies on oncological conditions, its implementations reach further. Currently, other indications merely contribute for 10% to the overall request for FP, including hematological autoimmune diseases such as systemic lupus erythematosus, gender dysphoria and critical illness (Yang et al., 2019). Furthermore, novelties in reproductive research in humans, livestock and laboratory animals are becoming more important for FP in precious domestic and wild animal species (Comizzoli, 2015).

### *5.2.1) OVARIAN TISSUE*

Nowadays, the cryopreservation of ovarian tissue of several thousand young women has been reported. Peer-reviewed publications have stated as many as 360 transplantations of frozen-thawed ovarian tissue (Andersen et al., 2019). Even though the vast majority claims restauration of both endocrine and fertility function (Kim, 2006; Smitz, 2010; Roness and Meirow, 2019), the survival span of the graft is variable (Roness and Meirow, 2019). So far, multiple studies have stated a pregnancy rate of 30% and higher and reported over 100 live births worldwide after ovarian tissue transplantation (OTT) (Jadoul et al., 2017; Andersen et al, 2019; Roness and Meirow, 2019). For the cryopreservation of ovarian tissue, there are two possible procedures that can be applied: slow freezing and vitrification (Bus et al., 2019). Slow freezing is used by most FP laboratories, basically applying a computer-assisted gradual freezing protocol, where samples are slowly frozen to -140 °C and subsequently plunged and stored in liquid nitrogen at -196 °C. One of the main challenges of cryopreservation of ovarian tissue is intracellular ice crystal formation and its accompanying tissue damage (Kim, 2006). For this reason, vitrification was developed as an alternative cryopreservation technique avoiding ice crystal formation by using an extremely high cooling rate. However, on the downside, concentrations of often toxic cryoprotectants need to be much higher (Kim et al., 2018). Although techniques have been enhanced

to avoid ice crystal formation, studies correlating slow freezing and vitrification showed inconsistent results (Bus et al., 2019). Both methods require thawing of cryopreserved tissue before being transplanted orthotopically or heterotopically (Rosendahl et al., 2013). The reported live births were mostly achieved following the transplantation of thawed slow-frozen ovarian tissue (Donnez et al., 2013). The transplantation of vitrified human ovarian tissue is still in its experimental phase (Kawamura et al., 2013; Suzuki et al., 2015). Nevertheless, taking a closer look at animal models, Kagawa et al. (2009) reported no loss of oocyte viability after auto-transplantation of vitrified-warmed tissue back to cattle-donors. Similar studies were conducted with ovarian tissue of mice (Migishima et al., 2003), baboons (Amorim et al., 2018), domestic dogs (Ackermann et al., 2017) and cattle (Beck et al., 2020), resulting in similar observations. Furthermore, the successful auto-transplantation and cryopreservation of whole rat (Yin et al., 2003) and sheep (Revel et al., 2004) ovaries have been reported. Even though encouraging results have been reported, cryopreservation of ovarian tissue in wildlife species still presents a huge challenge, mainly due to the biological variety of the cell types and a lack of standardized protocols (Campos et al., 2019).

Because OTT is performed without the creation of vascular anastomosis, there is a high risk of both ischemia and a considerable loss of PAFs (Kim et al., 2018; Roness and Meirow 2019). The various necessary steps conducted for OTC and OTT each contribute to a cumulative decrease in the number of viable follicles in different ways (Fig. 4), resulting in a loss of more than 70% (Donnez et al., 2004; Roness and Meirow, 2019). In theory, ischemia can be reduced by (1) decreasing hypoxic tissue damage with the aid of antioxidants or anti-apoptotic agents and (2) alleviating angiogenesis by enhancing the expression of angiogenetic factors (Kim, 2006). In the past, there have been various efforts to improve vascularization after transplantation, such as the use of VEGF during short term tissue culture prior to transplantation (Langbeen et al., 2016). Bovine cortical tissue strips that were exposed to VEGF *in vitro* were transplanted in immunodeficient mice. However, no beneficial effects of VEGF on the vascularization of the graft could be noted (Bus et al., 2019). Furthermore, multiple studies evaluated the advantages of orthotopic and heterotopic ovarian tissue auto-transplantation (Kim, 2006; Duncan et al., 2016). A pilot study conducted by Leonel et al. (2018) attempted to estimate the efficacy of peripheral auto-transplantation in cats. The grafts that were transplanted to

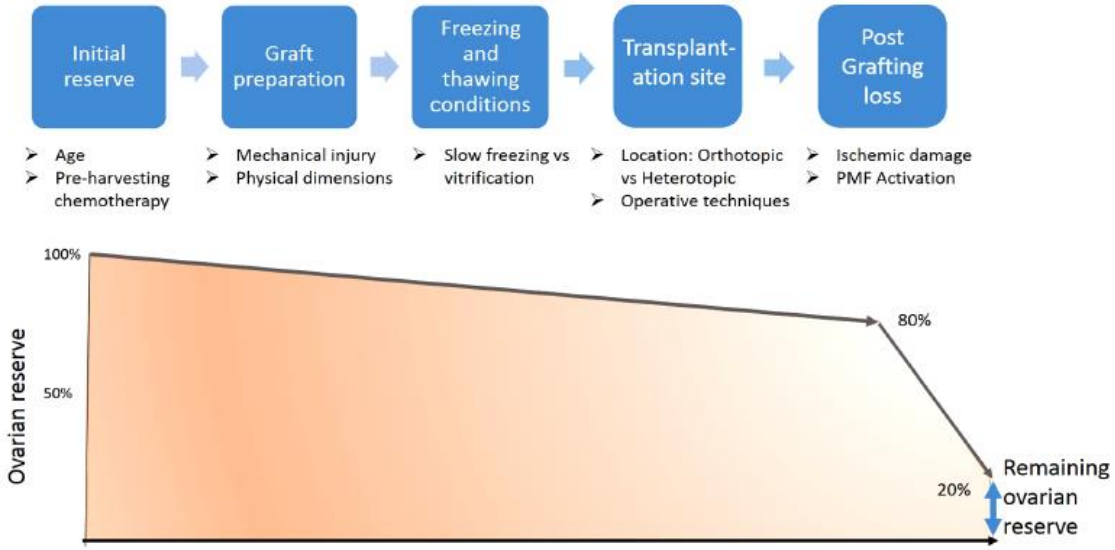


**Figure 4:** Loss of the ovarian reserve in the process of OTC and OTT. All of the steps, starting from retrieving the initial reserve and ending at the stage after grafting, can induce various hurdles that result in a significant loss of ovarian reserve (modified from Roness and Meirow, 2019).

subcutaneous tissue showed short- and long-term restoration of ovarian function including the production of hormones and growth of antral follicles. Indeed, a possible advantage of OTT can be the restoration of both the endocrine and fertility functionality (Smitz et al., 2010; Rosendahl et al., 2013). According to Smitz et al. (2010), a reason for the loss of PMFs after transplantation can be the uncontrolled follicular activation of the dormant pool. The authors demonstrated results of different studies where the proportion of growing follicles expanded from less than 20% before transplantation to up to 70% after grafting in humans, monkeys and sheep. This could be explained by a loss of inhibitory signals such as inhibin A, resulting in increased levels of FSH. Furthermore, significantly decreased levels of AMH could be the reason for this fast depletion.

One of the main contraindications for auto-transplantation of frozen-thawed ovarian tissue is the involvement of cancers that induce ovarian metastases. The transplantation of ovarian tissue derived from such patients induces a high risk on the re-introduction of malignant cells (Kim, 2006; Smitz et al., 2010, Rosendahl et al., 2013; Bus et al., 2019). However, it is not yet completely clear which types of cancer exactly disqualify the patient for OTC. In a review conducted by Rosendahl et al. (2013), the scientists detected "a lack of systematic studies evaluating the risk of malignant cell infiltration in ovarian tissue from patients with malignant disease who undergo OTC for fertility preservation".

### *5.2.2) ISOLATED FOLLICLES*

One major advantage of using isolated PAFs is that they cannot be invaded by malignant cells through the bloodstream due to their surrounding basement membrane that separates the PAFs from the ovarian stroma, any innervation, blood vessels and white blood cells (Rodgers et al., 2003). Therefore, the use of PAFs as part of a FP strategy can be a safer alternative for female patients with malignant types of cancer. Currently, there are two methods that are investigated more closely in human reproductive research: (1) the *in vitro* created artificial ovary and (2) the 'transplantable' artificial ovary (for review Bus et al., 2019). The first method requires the *in vitro* culture of ovarian tissue fragments with the sole purpose to complete the entire process of folliculogenesis of the included PAFs *ex vivo*. However, the complexity of folliculogenesis is – even today – not yet fully understood. This is why the second method could present a safer alternative as the isolated PAFs are auto-transplanted to their natural environment, embedded in and protected by an 'artificial graft'. Furthermore, patients in remission will have a higher chance to conceive naturally (Amorim, 2016). Out of all types of PAFs, primordial follicles tend to be the least vulnerable to cryoinjury (Silber, 2016; Jivago et al., 2018; Bus et al., 2019). According to Campos et al. (2019), these observations can be explained by certain characteristics that are typical for PMFs. These follicles only contain a limited quantity of intra-cytoplasmatic cold-sensitive lipid droplets, only hold a few organelles and have a relatively low metabolic activity. Moreover, PMFs lack cortical granules, a meiotic spindle and a zona pellucida. Also, the penetration of cryoprotectants is better in small sized follicles such as PMFs (Faustino et al., 2010). All of the above shows that PMFs are excellent cell types for long term FP, from a technical point of view.

Currently there are 2 techniques for the isolation of follicles from ovarian tissue that can also be combined: (1) mechanical and (2) enzymatic isolation. The first mechanical isolation protocols were used in the early 1990s and have been further enhanced ever since (Kim et al., 2016). Since primate ovaries mainly contain dense connective tissue, mechanical isolation has proven difficult (Campos et

al., 2019). On the other hand, mechanical isolation in cattle using a tissue chopper and microdissection has shown positive results based on an intact follicular structure and basal membrane as well as remaining morphological interactions between oocytes and the granulosa and theca cells (Campos et al., 2019; Bus et al., 2019). After many researchers had applied the isolation technique that was first described by Figueiredo et al. (1993), Langbeen et al. (2015b) described a new mechanical isolation method where ovarian cortex tissue is cut into small pieces, followed by blending and dispersion. Early PAFs isolated from the tissue of three ruminant species were classified immediately after retrieval and tested for viability by Neutral Red staining. Other morphological parameters such as follicular diameter and the quantity of granulosa cells can also be used for viability determination (Bus et al., 2019).

As stated above, the ovaries of most domestic species contain a high amount of dense connective tissue impeding follicular isolation. As a consequence, researches have been trying to find other ways to liberate the follicles from their surrounding tissue. The use of different types of enzymes, often combined, has proven quite promising. Collagenase, derived from *Clostridium histolyticum*, has been applied to isolate PAFs from murine, swine and bovine ovaries (Araújo et al., 2014). Different types of collagenase (Ia, II, IX, XI) are available (Dolmans et al., 2006). However, the enzyme might contain high endotoxin concentrations that have the ability to negatively interfere with culture and graft formation. Collagenase can either be used alone, in combination with mechanical isolation or combined with DNase to facilitate tissue handling in human (Lierman et al., 2015) and bovine fertility research (Wandji et al., 1996). In addition, this combination of enzymes was repeatedly described for the isolation of follicles in Australian wildlife, as the fat-tailed dunnart, the eastern and the northern quoll and the Tasmanian devil (Campos et al., 2019). In a study conducted by Shi et al. (2007), collagenase type I was used for enzymatic digestion of porcine ovarian tissue. Subsequently, a Percoll separation gradient was effectively used to ultimately improve the yield of PMFs. Liberase, a combination of purified enzymes, has proven its capability to enhance the quality of human pancreatic islet cell isolation. The cells displayed a better viability and an improved preservation of the anatomical integrity (Lierman et al., 2015). Liberase has also been used to isolate follicles in primates (Campos et al., 2019) and in humans (Dolmans et al., 2006; Lierman et al., 2015; Schmidt et al., 2018). PAFs can also be retrieved in some mammal donors by transvaginal and ultrasound-guided biopsy or by obtaining ovarian tissue post-mortem and therefor allowing the ovary to present as a continuable source of cortex tissue, holding viable PAFs (for review, Bus et al., 2019). According to Araújo et al (2014), studies have concluded a minimal to non-existing disruption of the ovarian function in cattle, horses and humans. This procedure can ultimately lead to a new way of recovering PAFs for *in vitro* maturation in genetically valuable species.

The acquirement of PAFs in most animals can be realized by transvaginal and ultrasound-guided biopsy or by obtaining ovarian tissue post-mortem and therefor allowing the ovary to present as a continuable source of cortex tissue holding viable PAFs (Bus et al., 2019). According to Araújo et al (2014), studies have concluded a minimal to non-existing disruption of the ovarian function in cattle, horses and humans. This procedure can ultimately lead to a new way of recovering PAFs for *in vitro* maturation in genetically valuable species.

## 5.3) SELECTION AND QUALITY ASSESSMENT OF GERM CELLS

The overview of female FP methods shows that even though numerous studies have been conducted, significant challenges still present. Looking at male FP and adjusting those techniques to female FP could be an option to minimize certain challenges.

### *5.3.1) SELECTION OF SPERM CELLS*

In Europe, approximately 20% of men suffer from impaired fertility of which only a fraction can ultimately be diagnosed with an underlying cause (Goossens and Tournaye, 2014). While FP in females usually comes with a high effort, most of the approaches in males are conducted more easily and are less costly. While one routine attempt of retrieving spermatozoa mostly delivers millions of germ cells, the logistical and medical effort necessary for the retrieval of the same quantity of viable oocytes is much more complicated. However, this only applies to adolescent males as their spermatogenesis has already started and thus allowing semen banking (Mitchell et al., 2009). The only possibility for prepubertal boys – similar to prepubertal girls – is the cryopreservation of testicular tissue. Unfortunately, this approach is still in its experimental phase (McBride and Lipshultz, 2018; Halpern et al., 2020). The same set of challenges applies to Klinefelter patients (Goossens and Tournaye, 2014).

Ultimately, the goal of FP is to produce viable offspring from proper genetic material. This is achieved with the use of ART, including artificial insemination (AI), IVF and intracytoplasmic sperm injection (ICSI) (Beydola et al., 2013; Chen et al., 2017). Most of the techniques used in ART require spermatozoa to be separated from the seminal plasma, as this is beneficial to sperm function but might hamper sperm survival (Grunewald and Paasch, 2012). *In vivo*, the spermatozoa separate from the seminal plasma rapidly after semen deposition and continue moving towards the oviduct (Bedford, 2008). Seminal plasma contains antioxidants to fight reactive oxygen species (ROS) and substrates such as fructose that are a source of energy for the spermatozoa, as well as decapacitation and sperm motility inhibiting factors (Mori and Sabanegh Jr., 2012). The latter is detrimental to *in vitro* sperm motility (Morrell and Rodriguez-Martinez, 2009), as are high concentrations of ROS since they initiate sperm membrane lipid peroxidation (Carrasquillo and Ramasamy, 2018). Nevertheless, a low concentration of ROS is thought to be essential to induce sperm membrane changes taking place right before fertilization (Aurich, 2005). Seminal plasma can also hold and transmit different pathogens to the female reproductive tract which is a commonly known problem in animal reproduction. The main pathogens include porcine respiratory and reproductive syndrome virus, bovine viral diarrhea virus, equine viral arteritis virus, *Ureaplasma urealyticum, Klebsiella* spp. and *Talorella equigenitalis* (Morrell and Rodriguez-Martinez, 2009). Another factor that plays a role in obtaining high quality sperm is the passive filtering system formed by the female reproductive tract *in vivo*. Whether the sperm needs to conquer both or only one barrier is determined by the ejaculate deposition site (Scott, 2000; Gadella and Luna, 2014). Ruminants and primates position the semen in the vagina, thus making the cervix their first hurdle. In contrast, the uterine tubal junction poses the first barrier to the semen of pigs, horses, camels and dogs as their semen deposition takes place in the uterus. This extends to species where sperm is deposited in the cervix or uterus with the application of AI, such as pigs, sheep, cattle and horses (Morrell and Rodriguez-Martinez, 2009).

Mechanisms considering both sperm quality and removal of seminal plasma *in vitro* have been and still are developed in the field of biomimetics. The devised techniques are categorized in 'separation' and 'selection' techniques (Table 1). The ultimate goal of the selection process is to isolate the most proficient sperm cells (Jeyendran et al., 2019). The most conventional and routinely available methods are illustrated in the following section.

**Table 1:** Biomimetic techniques for the separation and selection of spermatozoa (modified from Morrell and Rodriguez-Martinez, 2009)

| **Separation of Spermatozoa from Seminal Plasma** | **Separation from Seminal Plasma and Selection Based on Sperm Quality** |
|---|---|
| Washing | Migration<br>(swim-up, migration-sedimentation, swim-down) |
| | Filtration<br>(glass wool) |
| | Centrifugation on a colloid<br>(single layer, density gradient) |

### 5.3.1.1) Washing

The simple 'washing' method is one of the first separation methods ever developed (Grunewald and Paasch, 2012). The ejaculate is diluted in culture medium and centrifuged to separate the spermatozoa from seminal plasma. To reduce the risk of ROS formation by non-viable spermatozoa and leukocytes, the applied centrifugal forces are lower than 500 x *g*. Usually this technique is applied for the selection of spermatozoa used in intrauterine insemination (Beydola et al., 2013).

### 5.3.1.2) Migration

There are several migration techniques and all of them are based on the active motility of spermatozoa. This implies, that these methods are mimicking the separation from seminal plasma that spermatozoa perform *in vivo*. Hence, these techniques only work if the spermatozoa are motile (Morrell and Rodriguez-Martinez, 2009). The swim-up method (Fig. 5) relies on the self-propelled movement by using a cell pellet or a liquefied semen sample as the bottom layer, topped by sperm wash medium (Beydola et al., 2013). However, the procedure has been amended for oligozoospermic men, allowing the 'swim-up' to happen rather from semen than from the cell pellet (Jameel, 2008). It is one of the most commonly used techniques, even though this method can induce a relatively high

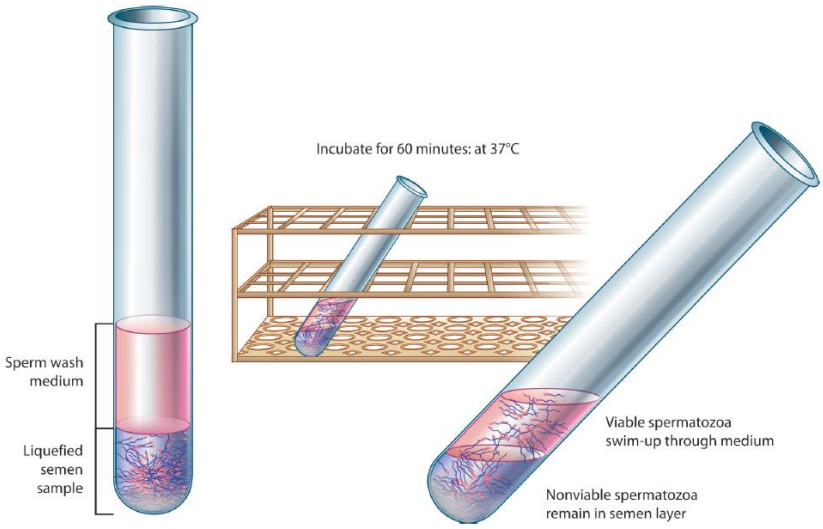


**Figure 5:** The swim-up technique (Beydola et al., 2013).

concentration of ROS (Beydola et al., 2013). In the case of low motility, migration-sedimentation can be used as it combines the swim-up method with the natural sedimentation of spermatozoa due to gravity (Mortimer, 1994). Furthermore, there is the swim-down technique which uses a gradient as a motility barrier (Beydola et al., 2013). None of those migration techniques is suited for the preparation of spermatozoa which will later be used in AI as they all have the predominant disadvantage of a low yield of spermatozoa in common (Morrell and Rodriguez-Martinez, 2009).

#### 5.3.1.3) Filtration

The filtration techniques require the spermatozoa to migrate through a column of either Sephadex or glass wool (Morell et al., 2017). The success of the process relies on both the motility and the interplay of the spermatozoa with the filter material (Mogas et al., 1998). The chemical and physical properties of glass and the thickness of the glass wool layer are directly correlated with the efficacy of the procedure (Grunewald and Paasch, 2012). The densely packed glass fibers catch the non-viable spermatozoa as well as debris and leukocytes which is essential as leukocytes are the main source of seminal ROS (Nani and Jeyendran, 2001). Since the separation from seminal plasma is achieved by centrifugation subsequently to the filtration process, the absence of non-viable spermatozoa and leukocytes decreases the ROS-production to a minimum (Beydola et al., 2013). Glass wool filtration has successfully been used in human (Sterzik et al., 1998; Rubessa et al., 2016), equine (Pessoa et al., 2017), procine (Bussalleu et al., 2009), bovine (Arzondo et al., 2012; Rubessa et al., 2016) and canine (Kim et al., 2010) sperm selection.

#### 5.3.1.4) Centrifugation

Density gradient centrifugation (DGC) separates motile, morphologically normal and chromatin-intact sperm cells from the semen sample (Fig. 6) which holds distinct subsets of spermatozoa (Morrell et al., 2017). Those subsets vary in their degree of maturation and therefore also in their density (Alvarez, 2012). The centrifugation through colloids takes advantage of those differences by placing every spermatozoon at the gradient level that complies with its density, known as their 'isopycnic point' (Morrell et al., 2016). A mature and morphologically normal spermatozoon has a density of at least 1.10 g/mL, while the density of an immature and morphologically abnormal spermatozoon ranges between 1.06 and 1.09 g/mL. The interfaces of the different gradients will hold white blood cells and debris (Beydola et al., 2013). There are 2 modifications of DGC in use based on the ultimate utilization of the selected semen in either AI, IVF or ICSI (Morrell et al., 2016).

Density gradients can be prepared either continuous, with density gradually increasing from the top to the bottom, or discontinuous (Beydola et al., 2013). The chosen gradient in the selection of spermatozoa for ART is discontinuous as it generates clear boundaries between the layers and has shown advantages regarding recovery and motility enhancement (Grunewald and Paasch, 2012). Through the last decades, different separation media such as Percoll® (Gadella and Luna, 2014), Ficoll® (Naeemipour, 2016), dextran-visotrast (Glander et al., 1996) and Nycodenz (Cartwright et al., 1991) have been extensively researched. The most widely used is Percoll®, a colloidal suspension of polyvinylpyrrolidone (PVP)-coated silica particles (Morrell and Rodriguez-Martinez, 2009). Despite the use of Percoll® in DGC substantially reduces ROS concentrations and the number of seminal bacteria, it carries a high risk of endotoxin contamination and can induce membrane alterations and inflammatory responses all of which could be transmitted to the oocyte with fertilization (Grunewald and Paasch, 2012). That is why Percoll® has been withdrawn from the market for use in human

reproduction and has been replaced by less toxic alternatives, such as PureSperm® or OptiPrep®, containing silane-coated silica particles (Kumar et al., 2019).

A simplification of the DGC is the Single Layer Centrifugation (SLC) in which only one layer of colloid is used (Morrell et al., 2016). In terms of preparing spermatozoa for AI, this technique is usually favored over the DGC, because it allows for larger volumes of semen to be processed in a shorter time (Crespo-Félez et al., 2017). SLC has already been used successfully in the retrieval of good-quality equine (Carlini et al., 2017), porcine (Crespo-Félez et al., 2017), dromedary camelid (Malo et al., 2017) and bovine (Goodla et al., 2014) semen. Furthermore, the use of SLC has been described in bears (Alvarez-Rodriguez et al., 2016), cats (Chatdarong et al., 2010), dogs (Dorado et al., 2013), red deer (Anel-Lopez et al., 2015) and llamas (Trassoras et al., 2012) – either prior to freezing to improve cryosurvival or after thawing to select the best quality sperm cells. The application of SCL after thawing is specifically useful when the spermatozoa are meant to be used in IVF or ICSI as those methods require only a small quantity of viable spermatozoa in contrast to routine AI (Morrell et al., 2016). Looking back at the challenges in the isolation of PAFs, the application of a density gradient seems to present a more viability sparing technique for germ cells.

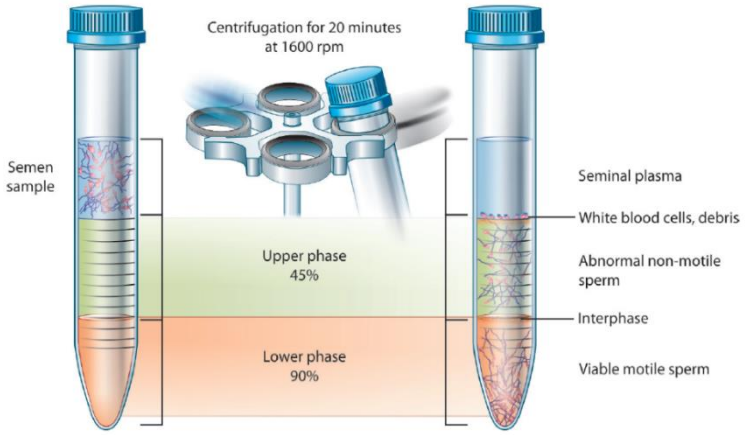


**Figure 6:** Density gradient centrifugation. The lower and upper phase of the gradient are carefully layered with the ejaculate put on top. The specimen is centrifuged for 20 minutes at 1600 rpm. The result is a 3-layered sample with clear seminal plasma at the top, followed by the interface containing white blood cells and debris. The second layer consists of abnormal non-motile sperm, while the third layer holds the viable motile spermatozoa. (Beydola et al., 2013)

### *5.3.2) QUALITY ASSESSMENT OF ISOLATED FOLLICLES*

As stated above, there are different possibilities to increase the yield of PAFs. However, the quality of those isolated PAFs should be considered. There are different ways to assess the quality of isolated follicles that will be illustrated more greatly in this section.

#### 5.3.2.1) Short term *in vitro* culture

Follicle growth and development are dependent on various underlying mechanisms such as autocrine and paracrine factors that regulate early folliculogenesis. *In vitro* culture can assist in the understanding of the follicular requirements supporting to follicle growth (Araújo et al., 2014). Furthermore, it allows the use of non-invasive parameters such as determining survival and follicle growth (Bus et al., 2019). Jorssen et al. (2015) used a short-term *in vitro* culture system of isolated individual PAFs to characterize follicular dynamics. During a 10-day culture, PAFs were evaluated regularly by microscopic assessment of the cell morphology and follicular diameter. Differences in the

connection between the oocyte and the surrounding (pre-) GCs as well as varieties in the integrity of the basal membrane led to a subdivision of the follicles into three categories (Fig. 7). The follicles with the most optimal morphological features (see below) were allocated to category 1. Almost 70% of all cultured PAFs survived the full culture period, in which an intact basal membrane and connection between the oocyte and its GCs appeared to be determining factors qualifying the follicles for further development. Moreover, a correlation between the enlargement of the follicular diameter and the increase in the total cell number was reported. In other words, category 1 follicles showed better quality than category 2 and 3 follicles, as their morphological features allowed a better developmental capacity *in vitro* (Jorssen et al., 2015).

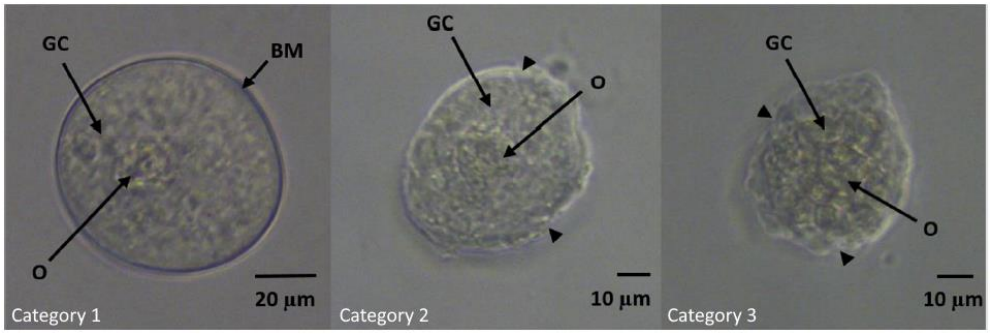


**Figure 7:** Category 1 follicles contain an intact basal membrane (BM) and an intact connection between the oocyte (O) and the surrounding granulosa cells (GC). Category 2 follicles display a torn basal membrane (arrowhead), while the connection between the oocyte and the granulosa cells is still unimpaired. Category 3 follicles have both a disrupted basal membrane and lack contact between granulosa cells and the oocyte. (Modified from Bus et al., 2019).

#### 5.3.2.2) Neutral red staining

Neutral red (NR) is a non-toxic dye that has the ability to stain lysosomes (Schmidt et al., 2018). The uptake of the dye depends on the cell's capacity to sustain gradients through the production of ATP. At a physiological pH, the net charge of the dye is close to 0 and thus enabling it to penetrate the cell membrane. Lysosomes are acid cell structures that use a proton gradient to keep their pH lower than the cytoplasmatic pH. Once the dye has entered the cell structure, it gets charged and therefor remains in the organelle (Repetto et al., 2008). Dying cells are progressively losing the capacity to incorporate the stain. Therefore, NR can be used to visualize viable follicles (Schmidt et al., 2018). NR can be used to evaluate follicle content, the viability in cortical tissue strips and for the staining of isolated follicles (Fig. 8). Multiple studies have established that NR neither affects the enzymatic activity within the cell structures, nor results in negative effects on the follicles mid-term capacity to grow (Kristensen et al., 2011; Langbeen et al., 2014; Jorssen et al., 2014).

#### 5.3.2.3) Calcein staining

Calcein (Blue) AM, a cell-permeant fluorescein-derivate, can be applied to establish the cell's viability in most eukaryotic cells (Nirmala and Lopus, 2019). After entering a living cell, the non-fluorescent calcein AM is cleaved by esterase and therefor converted into a fluorescent calcein, resulting in a uniform green or blue fluorescence (Fig. 8) (Lierman et al., 2014). After exposing to light with a wavelength of 360 nm and observing the emitted light at a wavelength of 449 nm, the blue fluorescence can be visualized. For the green fluorescence, the wavelengths are supposed to be 495 and 515 nm. The viability of follicles can be assessed within one hour (Bus et al., 2019).

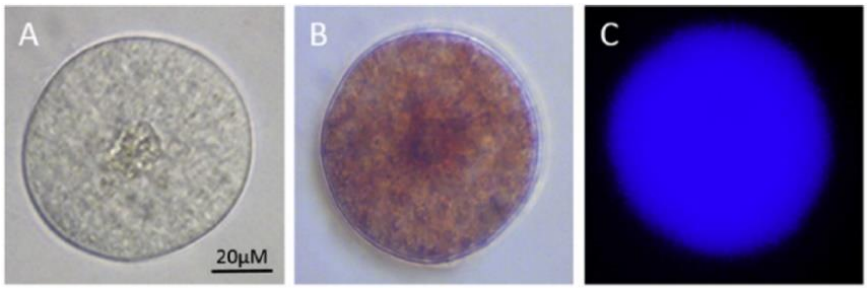


**Figure 8:** Follicles stained with NR and Calcein Blue AM to show the viability of follicles on light microscopy. A) Non-stained follicle. B) Positively NR stained and thus viable follicle. C) Positively calcein stained and thus viable follicle (Modified from Bus et al., 2019).

#### 5.3.2.4) Gap junction identification and assessment of functionality

The main connexins expressed in the female reproductive organs are Cx37 and Cx43. Those two gap junction proteins seem to be crucial to normal folliculogenesis (Kidder and Vanderhyden, 2010). The functionality of connexins can be evaluated by injecting Lucifer Yellow into the oocyte. If the connexins are functional, the fluorescent dye will get transferred from the oocyte to its surrounding GCs (Ali et al., 2005). As cryopreservation of PAFs could be used as an alternative to embryo cryopreservation and mature-oocyte cryopreservation after ovarian stimulation, gap junction identification could be a possible tool to document follicle viability before and after thawing (Bus et al., 2019). One of the challenges in developing a successful strategy for cryopreservation of PAFs is that vitrification can cause cryoinjuries. This can lead to a damage of membrane proteins, such as connexins (Bus et al., 2019). Vitrifying bovine blastocysts seems to induce the opening of usually closed connexons. Commonly, connexons only open when exposed to stress. Consequently, blocking connexons can protect embryos from temperature induced injuries (Oritz-Escribano et al., 2017). On that account, displaying functionality of the connexins before and after vitrification plays an important part in assessing the viability and the consecutive developmental capacity of follicles after cryo-treatment.

#### 5.3.2.5) Xenotransplantation

The developmental capacity of follicles has been assessed in various studies using animal models for xenotransplantation (Soares et al., 2016; Kim et al., 2018). The xenotransplantation of ovarian tissue from different species (e.g. baboons, elephants, wallabies and lionesses) to immunosuppressed rodents resulted in simple activation of PMFs and growth until the early antral stage (Campos et al., 2019). In a study, that transplanted human ovarian tissue to nude mice, the grafting sites where evaluated more closely. The peritoneum, ovarian bursa, subcutaneous and muscular tissue were all found to be equally capable of safeguarding the pool of dormant follicles and enabling early follicular growth. Nevertheless, the developmental capacity of embryos seemed to be lower in fertilized oocytes collected form grafted ovarian tissue (Soares et al., 2016). While xenografting with human tissue is still experimental, several experiments have been conducted in wild mammals, such as felines and non-human primates (Campos et al., 2019). However, Kim et al. (2018) stated a few noteworthy concerns regarding xenotransplantation. Even though high rates of follicular survival have been seen after transplantation, abnormal microtubule organization and chromatin patterns emerged during the maturation process, possibly resulting in aberrant oocytes. Furthermore, the risk of trans-species infections with prions or retroviruses among others should also be assessed.

# 6) AIM OF THE THESIS

In the last decade, the interest in different approaches to fertility preservation has been increasing profoundly. Not only the knowledge to the causes of infertility has been enhanced, but also new scientific and biotechnical approaches to fighting those causes have been gathered and studied by researchers around the world. While in humans, the increasing success in cancer treatment strategies in both adults and prepubertal boys and girls is the main driving force, the resident threat of climate change to different species pushes scientists to broaden their approaches in animals. Among others, the continually growing human population needs more resources and land. This ultimately leads to a reduction in space for wildlife species, which makes the preservation of endangered species an important task. Furthermore, there is an increased demand for nourishment. Selection of livestock that has favorable genetical treats, e.g. a profitable food conversion rate to increase the efficiency of the food-to-wanted-output conversion, can support this demand. This way, more meat or milk can be produced per animal, at a lower overall cost. Preantral follicles have been in the focus of studies on female fertility preservation. Ethical issues and an overall meager availability, however, induce a scarcity in human tissue obtainable for further research. Regarding the similarity in ovarian size, structure and function including oocyte characteristics, the bovine model has become a welcomed gateway for human and animal fertility preservation research. Due to the unlimited access to bovine ovaries from slaughterhouses, this model is an ideal option for reproductive research.

Even though approaches and techniques have been investigated broadly, there still are limitations and a lack of knowledge in the many subfields of fertility preservation. The isolation of PAFs is one of those branches. Various approaches have been studied to ensure isolation that results in the greatest yields of follicles, preferably of high quality. Although different strategies are available, there is no 'golden standard protocol' available yet, which displays that there is a need for optimalization. One method that has been evaluated by only a few studies, is the isolation of follicles through a density gradient. This approach actually originates from the selection of sperm cells and thus from male fertility preservation.

The aim of this study is the optimization of the *in vitro* isolation of preantral follicles from ovarian cortical tissue. After the application of the follicle retrieval protocol that has already been used in the 'Gamete Research Center' of the Laboratory for Veterinary Physiology and Biochemistry (University of Antwerp), the suspension including the follicles is separated by a discontinuous Percoll® gradient into different layers. The idea is to investigate each layer and to determine:

(1) the viability of the isolated follicles by the use of Neutral Red staining and

(2) the actual yield of viable and non-viable follicles in each layer.

# 7) MATERIAL AND METHODS

## 7.1) COLLECTION AND PREPARATION OF THE OVARIAN TISSUE

Bovine ovaries were collected at a slaughterhouse in batches of 20-100, depending on the number of animals presented for slaughter. The adnexa and mesovarium of the ovaries were removed with a pair of scissors. Within two hours after collection, the ovaries were transported to the laboratory in physiological saline (NaCl 0,9%; B. Braun) at 25°C. Upon arrival, the ovaries were washed in warm (37°C) physiological saline supplemented with 0.25% kanamycin in previously sterilized beakers. Subsequently, they were rinsed with 70% alcohol and washed twice in physiological saline at 37°C supplemented with kanamycin. After puncturing the macroscopically visible antral follicles for different *in vitro* research, five ovaries without a corpus luteum and no or little scars were selected.

## 7.2) MECHANICAL CORTEX ISOLATION

All tools that will be described in the following sections were either previously sterilized in a dry heat oven or autoclave or have been purchased in sterile packaging. Per ovary, one or more grit patterns with a total surface of 2 $cm^2$ were carved into the cortex utilizing a no. 26 surgical blade on a no. 4 handle, aiming to retrieve cortical fragments of approximately 1 $mm^3$ (Fig. 9). The scalpel was moved parallel to the ovarian surface, cutting small fragments of approximately 1 mm in thickness. The sliced tissue was then transferred into a 50 ml self-standing conical tube, containing 10 ml of filtered TCM isolation medium.

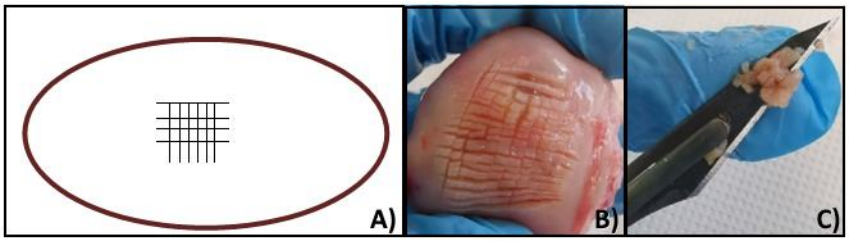


**Figure 9**: Mechanical ovarian cortex isolation used for retrieving small tissue fragments. A) Schematic illustration of the applied grit pattern. B) Grit pattern of approximately 2 $cm^2$ on a bovine ovary. C) Cortical fragments of circa 1 $mm^3$.

After filling the tube with cortical fragments up to the 5 ml mark, the fragments were mixed with the disperser (T 18 basic ULTRA-TURRAX®, IKA, Germany), 2 minutes with a large mixer (14 mm diameter; 3451600, S18D-14-KS) and 1 minute with a small mixer (10 mm diameter; 3451600, S18D-10G-KS) (Fig. 10).

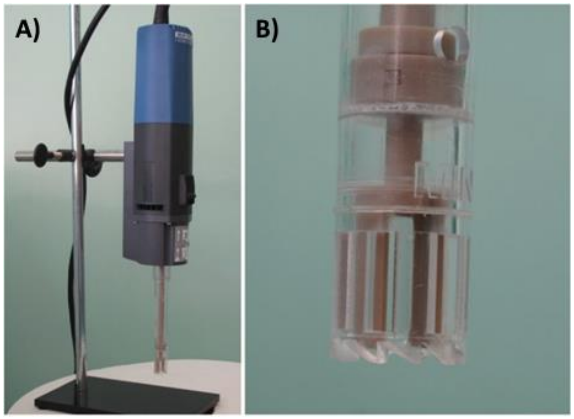


**Figure 10**: Disperser for grinding ovarian cortical fragments into a homogenous suspension. A) Disperser. B) Mixer with an inner rotor that moves with a maximum circumferential speed of 12 m/s. (Modified from Langbeen et al., 2015b)

## 7.3) FOLLICLE COLLECTION

After the homogenization process, the self-standing conical tube containing the suspension was moved to a laminar flow cabinet. All following procedures were conducted on the hotplate (38.5°C) in the cabinet to ensure the survival of the temperature-sensitive follicles and to prevent contamination. The received homogenous follicle suspension was filtered through a 100 µm filter (VWR 352360) into a 50 ml conical tube. To prevent the filter from clogging, a sterile blue pipette tip was scratched over the filter surface while moving the filter up and down. As the dispersion of ovarian tissue results in foam formation, the 100 µm filter was replaced by a new strainer halfway through the filtration process. Furthermore, the filters were rinsed with TCM isolation medium to ensure that no follicles were left behind. The suspension was filtered once again through a 70 µm mesh filter (VWR 352350) in another 50 ml tube while constantly moving the strainer up and down. The last filtering process was done with a 40 µm mesh filter (REF 352340) in a new 50 ml tube following the same procedure as described above. The resulting suspension was then distributed from the 50 ml tube into two conical tubes of 15 ml. Both tubes were centrifuged at 50 x *g* for 5 minutes at room temperature. This resulted in a separation of precipitated pellet and the superincumbent supernatans (Fig. 11). Subsequently, the supernatans was discarded with a Pasteur pipet. The pellet was further processed as described below.

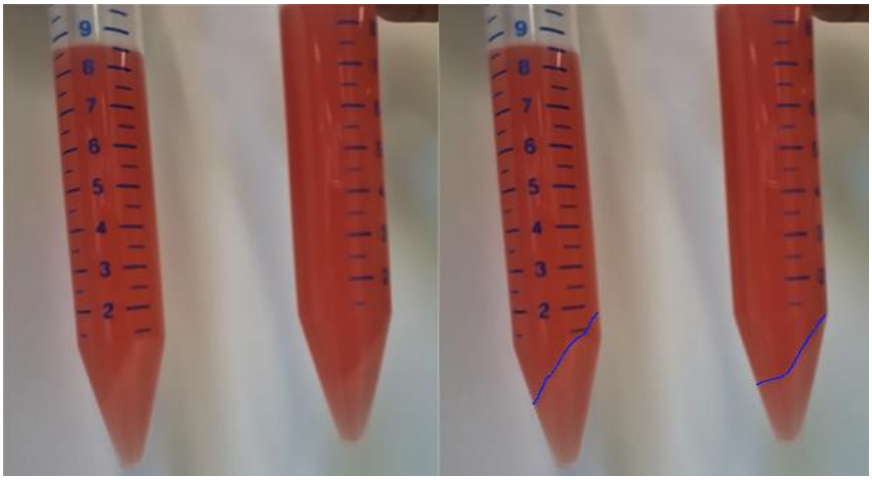

**Figure 11:** Visible separation of the pellet containing the follicles and the overlying supernatans after the centrifugation of the filtered suspension at 50 x *g* for 5 minutes. The right photo is a replica of the left one, indicating the line between the pellet and the supernatans.

The choice of the filter diameters (Table 2) was mainly based on existing literature (Rosales-Torres et al., 2012) and research performed at our laboratory (Gamete Research Center, Laboratory for Veterinary Physiology and Biochemistry, University of Antwerp) by Langbeen et al. (2014).

**Table 2:** Comparison of the mean bovine PAF diameter between different authors.

| Author | Mean primordial follicle diameter | Mean primary follicle diameter | Mean secondary follicle diameter |
|---|---|---|---|
| Rosales-Torres et al., 2012 (Review) | ≤ 40 µm | 40 – 80 µm | 81 – 130 µm |
| Langbeen et al., 2014 | 38.6 ± 4.6 µm | 47.4 ± 5.5 µm | 65.0 ± 12.5 µm |

## 7.4) PREPARATION OF THE STOCK SOLUTIONS

All stock solutions were prepared in a laminar flow cabinet prior to the follicle isolation process.

### *7.4.1) TCM ISOLATION MEDIUM*

A total of 50 ml TCM isolation medium was prepared in a 50 ml conical tube. The TCM199 (Fisher-Scientific, 31150) was enriched with 0,23 mM sodium pyruvate (Life-Technologies, 11360-039), 22mM Hepes buffer (Sigma-Aldrich, H6197) and 5% Fetal Bovine Serum (Sigma-Aldrich, F9665). Furthermore, Penicillin-Streptomycin (200 µg/ml; Life-Technologies, 15140-122) was added to the solution to prevent bacterial growth. The obtained TCM isolation medium was mixed and filtered through a 0,2 µm nylon filter into two new 50 ml tubes and refrigerated at 4°C. Two hours before the medium was used, two conical tubes of 10 and 15 ml were filled with the TCM isolation medium and placed in the incubator (38.5°C; 5% $CO_2$).

### *7.4.2) PERCOLL® GRADIENT SOLUTIONS*

The different density layers for the preparation of the discontinuous Percoll® (Sigma Aldrich, P1644) gradient were prepared based on the Ficoll densities suggested and investigated by Martinez-Madrid et al. (2004). As part of the gradient solutions, 10 ml of 1,5 M NaCl was prepared using sterile water (B. Braun). For each Percoll ® concentration, 10 ml of gradient solution were prepared. For the layer of Percoll® with its own density (1.1 g/ml), 20 ml were prepared. Table 3 displays the volume and composition of the different compounds used for the three distinct Percoll® gradient solution concentrations.

**Table 3:** Protocol for the preparation of the three different density gradient solutions with Percoll®. A final volume of 20 ml was prepared for solution 1 (1.1g/ml). Of solutions 2 (1.09 g/ml) and 3 (1.06 g/ml) respectively 10 ml is prepared.

| | Product | Total volume | Final concentration |
|---|---|---|---|
| **Percoll® gradient solution 1 at 1.1 g/ml density** | **1,5 M NaCl** | 2 ml | 1,5 M NaCl |
| | **Percoll®** | 14,60 ml | 1,1 g/ml |
| | **Sterile water** | 3,40 ml | - |
| **Percoll® gradient solution 2 at 1.09 g/ml density** | **1,5 M NaCl** | 1 ml | 1,5 M NaCl |
| | **Percoll®** | 6,53 ml | 1,09 g/ml |
| | **Sterile water** | 2,47 ml | - |
| **Percoll® gradient solution 3 at 1.06 g/ml density** | **1,5 M NaCl** | 1 ml | 1,5 M NaCl |
| | **Percoll®** | 4,20 ml | 1,06 g/ml |
| | **Sterile water** | 4,80 ml | - |

## 7.5) RETRIEVAL OF ISOLATED FOLLICLES BY A DISCONTINOUS PERCOLL® GRADIENT

The pellet containing the follicles was resuspended in 4.0 ml of Percoll® gradient solution 1 (Table 3; density = 1.1 g/ml) at the bottom of a 15 ml conical tube using a sterile blue pipet, creating the first Percoll® density layer. Subsequently, the following density layer solutions were added on top of the first layer using a serological pipet: 2.0 ml of 1.09 g/ml Percoll® solution (Table 3), 1.3 ml of 1.06 g/ml Percoll® solution (Table 3) and as a final layer 1.3 ml of TCM isolation medium.

As there were two tubes containing pellet, the same procedure was repeated with the second pellet. The resulting two gradient tubes were centrifuged at 50 x *g* for 17 minutes at room temperature to finally reach the precipitation and separation of the pellet components into their corresponding density interfaces (Fig. 12). While the fluid layers of the 1.06 g/ml and the 1.09 g/ml were transferred into a 50 ml beaker using a micropipette, all pellet layers were cautiously pipetted through slowly circling the tip of the micropipette along the fringe of the tube and transferred into a separate sterile 60 mm Petri dish. Also, the TCM-layer and the 1.1 g/ml density layer were each transferred into a sterile 60 mm Petri dish. For the ultimate assessment of survival and immediate viability of the follicles 0,2 µL of filtered Neutral Red Stain was added to each Petri dish. The stain was homogenously spread by carefully swaying the Petri dishes in circles. Finally, the Petri dishes were covered by a cardboard lit to protect the photosensitive cells from light and incubated for 20 minutes at room temperature.

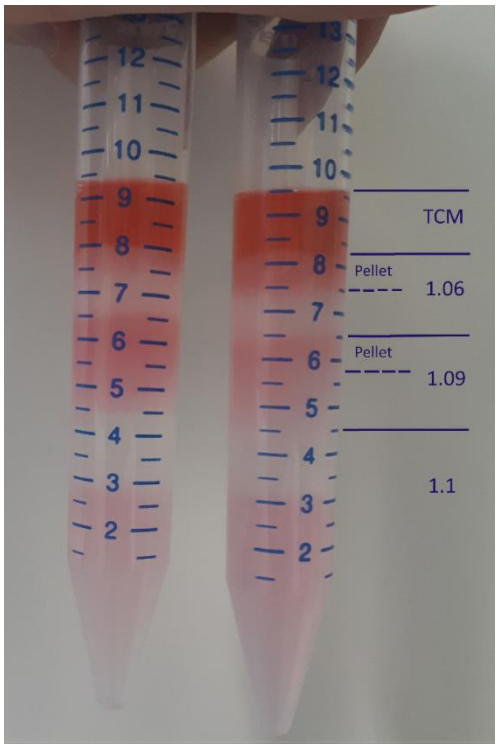


**Figure 12:** Presentation of the discontinous Percoll® gradient after centrifugating the tubes at 50 x *g* for 17 minutes at room temperature. The layer with a density of 1.1 g/ml is situated between 0 and 4.5 ml, the 1.09 g/ml density layer is placed between 4.5 and 6.5 ml and the 1.06 density layer is located between circa 6.5 and 8 ml. The pellet layers can be found between approximately 5.5 and 6.5 ml and 7.5 and 8.5 ml.

# 8) RESULTS

## 8.1) RETRIEVAL OF ISOLATED PAFs BY THE USE OF A DISCONTINOUS PERCOLL® GRADIENT

The mechanical follicle isolation combined with the discontinuous Percoll® gradient method for the retrieval of isolated bovine preantral follicles resulted in a total of 2,360 preantral follicles. Approximately 33% of the recovered follicles were categorized as large follicles, constituting early primary follicles. The residual 67% of the follicles were labeled as small follicles, corresponding to primordial follicles. No follicles were found in the 1.1 g/ml density layer. The largest percentage of follicles was found in the 1.09 Percoll® layer, with 53.18% (n = 1255). The 1.06 Percoll® layer consisted of 972 follicles, corresponding to 41.19% of the total number of follicles. The TCM isolation medium contained 5.63% (n = 133) of the follicles.

## 8.2) THE EFFECT OF PERCOLL® ON FOLLICULAR VIABILITY

The viability of the follicles was assessed by the use of Neutral Red Staining (Fig. 13). A follicle was classified as positively stained and therefor viable when the oocyte and at least 75% of the granulosa cells colored red. The follicles were classified into four categories: (1) large/stained, (2) large/non-stained, (3) small/stained and (4) small/non-stained. Follicles categorized as large, had a diameter of approximately 25 – 40 µm, while small follicles had a diameter < 25 µm. An inverted microscope (Olympus CKX 41 – magnification: 100 – 200x) was used to find the follicles.

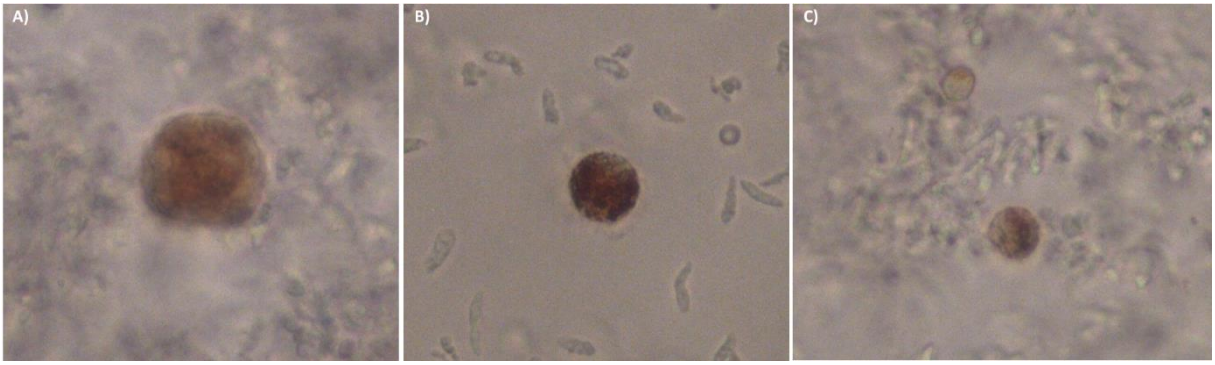


**Figure 13:** Display of different follicles types seen with an inverted microscope with a magnification of 100x. A) Large and stained follicle with a diameter of 34,45 µm. B) Small and stained follicle with a diameter of 17,68 µm. C) Small and non-stained follicle on the top and small and stained follicle (diameter = 13,38 µm) at the bottom.

Out of the 2,360 follicles, 72.75% were found to be viable (n = 1717). The non-viable fraction was discovered to be 27.25% (n = 643). Table 4 shows the distribution of follicles in the different layers and categories. While the most viable early primary follicles were situated in the 1.06 g/ml Percoll® layer, the most viable primordial follicles were located in the 1.09 g/ml Percoll® layer. With 52.54%, the small/viable follicles formed the largest part of the isolated follicles. The greatest standard deviation manifested in the 1.09 g/ml Percoll® layer (s = 172,43), as well as the largest mean number of follicles ($\overline{x}$ = 199,5; Fig. 14). The mean number of follicles found in the TCM layer are 15,5, while in the 1.06 g/ml Percoll® layer 95 follicles were counted on average.

**Table 4:** Distribution of the follicles divided in four categories. The stained follicles are representative for viable follicles. The non-stained follicles show the number of follicles that are classified as not viable.

| Layer | Large/stained | Large/non-stained | Small/stained | Small/non-stained |
|---|---|---|---|---|
| TCM isolation medium | 21 | 19 | 62 | 31 |
| 1.06 g/ml Percoll® | 308 | 145 | 380 | 139 |
| 1.09 g/ml Percoll® | 148 | 130 | 798 | 179 |
| Total n = 2,360 | 477 | 288 | 1,240 | 349 |

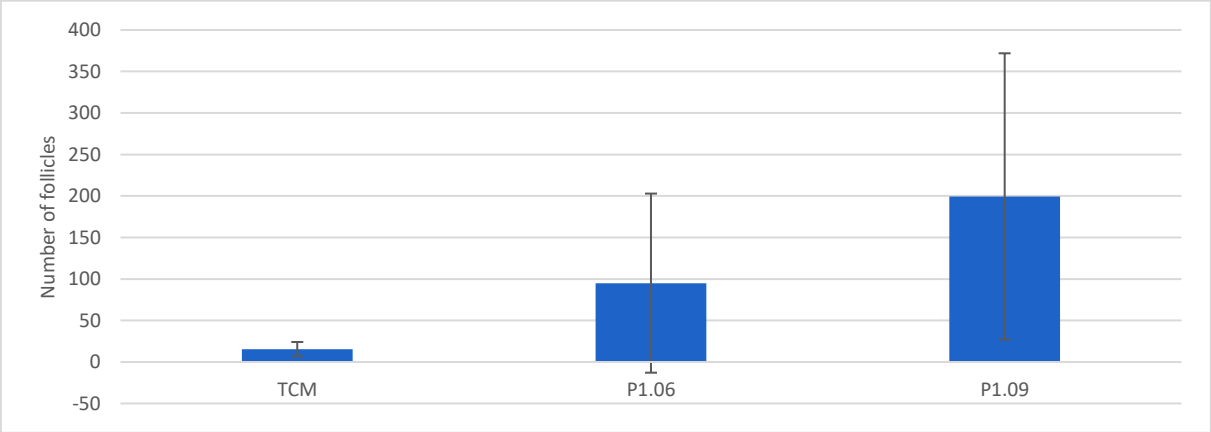


**Figure 14:** Distribution of the total number of follicles found in the different Percoll® density layers: TCM isolation medium, 1.06 g/ml Percoll® and 1.09 g/ml Percoll®. While the number of follicles rises proportionally with the increase of the Percoll® density, the standard deviation rises likewise.

Taking a closer look at the distribution of the different categories in every separate layer, the greatest spread of follicle numbers was seen in the 1.09 g/ml Percoll® layer, more specific in the number of small and stained follicles ranging from 13 to 432 (Fig. 15). The greatest outliers were found in follicle categories 1, 3 and 4 in the 1.06 g/ml Percoll® layer (Fig. 16). The TCM isolation medium layer contained the least follicles (Fig. 17), where category 3 follicles represented the largest group (n = 62).

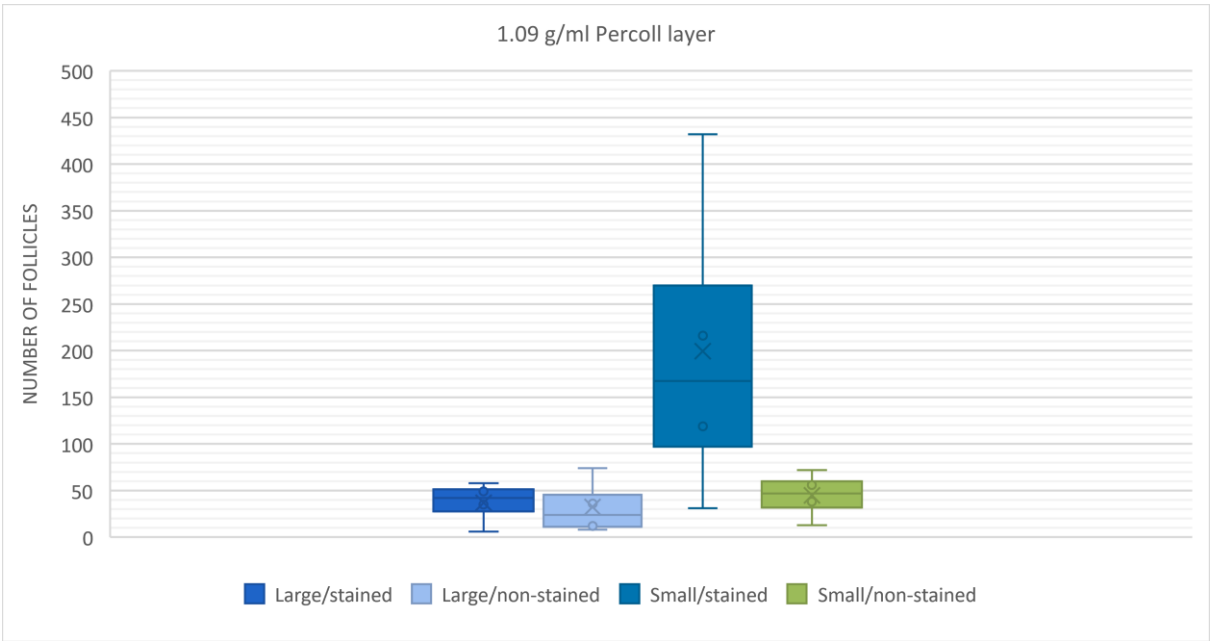


**Figure 15:** Distribution of the different follicle categories in the 1.09 g/ml Percoll® layer. In total, this layer contained the most follicles in comparison with the other layers. Also, the category 3 follicles (small/stained) where present in the largest quantity.

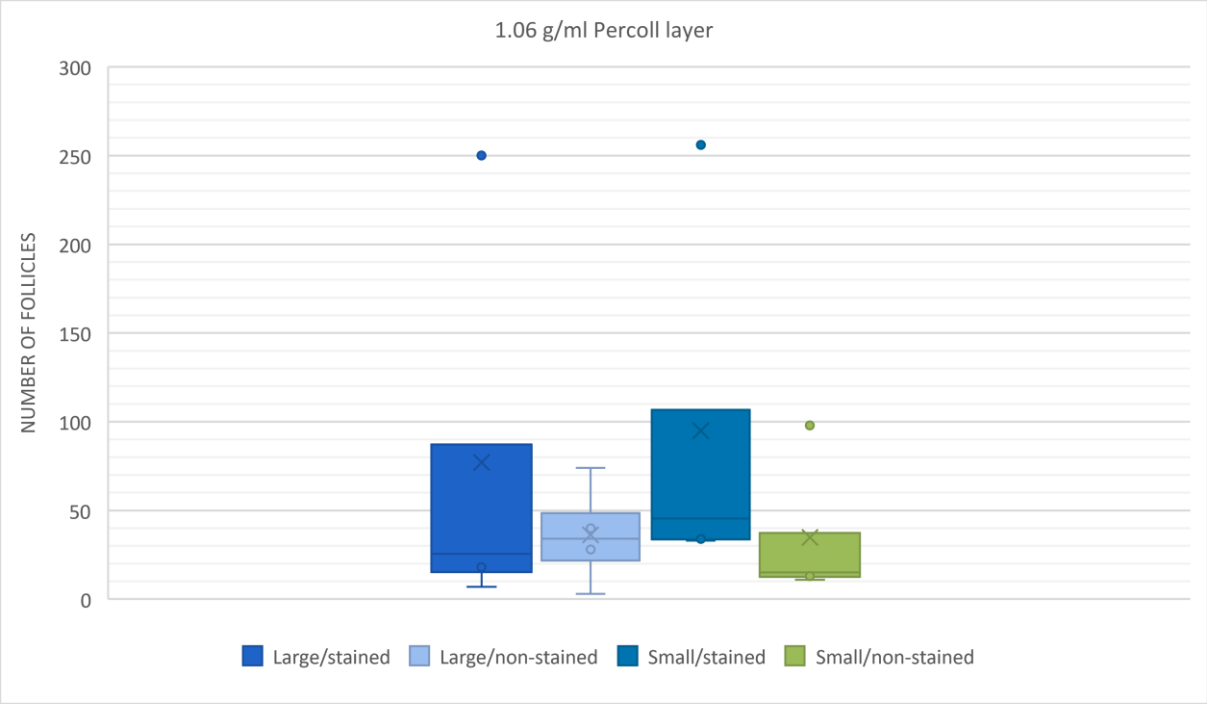


**Figure 17:** Distribution of the different follicle categories in the 1.06 g/ml Percoll® layer. The outliers are the most noticeable fact that presented in this layer. Also, the broadest ranges of follicles were represented by category 1 and 3 follicles, meaning that this layer contained the largest quantity of viable follicles compared to the total number of follicles present.

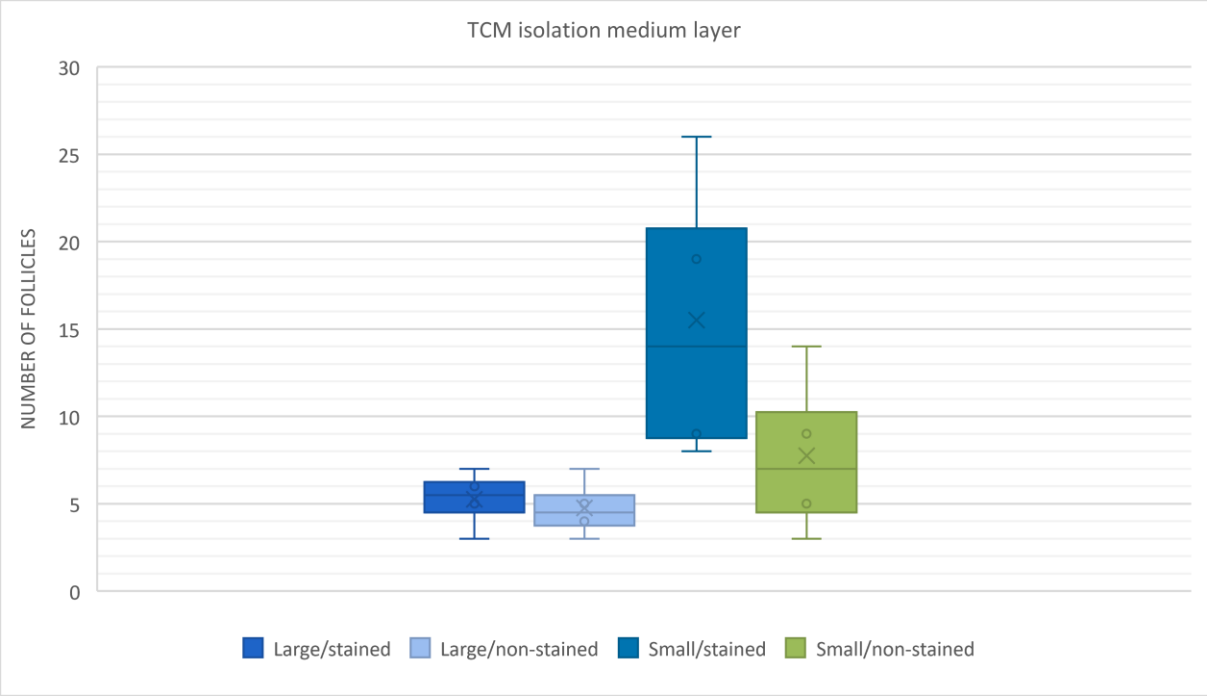


**Figure 16:** The TCM isolation medium layer included the smallest quantity of follicles with the most follicles out of category 3 and the least number in category 2.

# 9) DISCUSSION

In the past decades, various methods for the isolation of germ cells have been investigated in the field of reproduction and fertility preservation. In humans, the loss of the reproductive capacity due to aggressive cancer treatment, pathologies that affect the reproduction tract and gender dysphoria are the main indications for theses FP techniques. The growing list of endangered species as a result of climate change, the human contribution to the diminution of unimpaired habitats and the need for livestock with favorable genetic traits to ensure nourishment for the increasing human population are the main driving forces for the FP in animals. Researches have tried to find the best model in different species, e.g. marsupials, livestock and rodents, that can be applied to a broader group. Even though numerous studies have been conducted, there is no 'golden standard protocol' yet for the germ cell isolation process itself as it includes many intermediate steps that can be modified. This displays the need for the optimalization of isolation protocols.

The different approaches in males have been explored extensively with promising results. Techniques that consider both sperm quality and the removal of the mostly toxic seminal plasma have been developed. Nevertheless, ROS formation still forms a serious threat to sperm quality with all the non-advanced techniques. One of the main differences to female germ cell isolation is the much lower logistical, financial and medical effort needed to retrieve the same amount of germ cells in one isolation process. Furthermore, the male germ cell stock is nearly infinite. In females, the ovarian cortex contains a limited number of follicles. The PMFs are the main representatives of this group and are therefore focused upon in FP. The ultimate goal is to gain a maximum yield of viable follicles that eventually can be processed *in vivo* by transplantation or *in vitro* by culture. The cryopreservation of ovarian tissue has shown promising results so far. However, the steps needed to be taken starting from the actual harvesting up to the post grafting state result in a loss of approximately 80% of the initial ovarian reserve (Roness and Meirow, 2019). OTT and OTC are the only FP options available for female prepubertal cancer patients. Nevertheless, OTT and OTC can only be applied in patients that do not suffer from cancers that induce ovarian metastases as it includes a high risk for reintroduction of cancer cells (Rosendahl et al., 2013). The current understanding of OTC and OTT shows an upward trend and has the possibility to give hope to prepubertal cancer patients, but research is not yet fully exhausted. Challenges as ischemia and survival after transplantation need to be determined more precisely and solutions need to be found. That is one of the reasons for the increased interest in the isolation and preservation of PAFs. It is known that PAFs cannot be invaded by malignant cells through the bloodstream owing to their surrounding basal membrane. On that account, models for an *in vitro* artificial ovary and a transplantable artificial ovary have been investigated. One of the major concerns of the artificial ovary though is that the entire folliculogenesis is accomplished *ex vivo* which can result in serious consequences. The transplantable ovary could form a safer alternative with the great advantage that patients would get the chance to conceive naturally. However, both systems are not optimized yet and are therefore still more theoretical than practical applicable.

As of today, there are two possible routes to be taken in the isolation of PAFs: the mechanical or the enzymatic isolation. Both methods come with a great variability in the protocol steps, e.g. the availability of various enzymes. Depending on the species, the ovarian cortex contains a certain amount of connective tissue making either the mechanical or the enzymatic procedure more applicable. There are various protocols in both the mechanical and the enzymatic isolation that can be followed leading to a variety of combination possibilities. The steps following the actual isolation

process are utterly diverse. Due to this variability there are nearly unlimited possibilities for the combination and correlation of these different steps. Therefore, the study conducted in this thesis intended to contribute to the optimalization of a routinely applicable protocol for the retrieval of isolated preantral follicles. The method presented in this study therefor utilizes the Percoll® discontinuous density gradient separation method which initially derived from sperm cell isolation protocols and had already been modified for the isolation of human (Martinez-Madrid et al., 2004) and porcine (Greenwald and Moor, 1989) follicles. In this study, bovine ovaries were used because of the proven similarities between the bovine and the human reproductive pre-implantation physiology as well as the unlimited access to bovine slaughterhouse ovaries.

After the mechanical isolation of the follicles out of the ovarian cortex, the homogenous follicle suspension was filtered to remove tissue fragments that would disturb the ultimate examination and counting of the retrieved follicles. However, tissue pieces smaller than 40 µm went through the filter together with the follicles leading to a light impureness of the layered follicle-Percoll®-suspensions. Out of 2,360 counted follicles, 72.75% of the follicles were classified as viable after the viability staining with Neutral Red. Compared to the data collected by Martinez-Madrid et al. (2004) who had a viability score of 95.8%, this resulting percentage is relatively low. Sixty-seven percent of the recovered follicles had a diameter smaller than 25 µm, measured with an inverted microscope, corresponding to small primordial follicles according to published data (Table 2), while 33% had a size of 25 – 40 µm. The 1.09 g/ml Percoll® layer showed the highest yield of follicles out of all the layers investigated. With every test conducted, there were two test-tubes that were investigated. A recurring significant issue was that the batches that where investigated second had a smaller number of viable follicles. This leads to the conclusion that the viability of the follicles despite all the protecting measures taken is strongly time sensitive.

Looking at future perspectives, a discontinuous density gradient can be a good option to sort and retrieve follicles if enough ‘manpower’ is available. The processing time is crucial to follicle viability and therefore needs to be kept as short as possible. In human research, Percoll® is not used anymore due to its adverse effects on the oocyte and embryo quality. Further investigations need to be conducted to determine if those same adverse effects apply to bovine germ cells. Luckily, there are less toxic alternatives available. When isolated mechanically, follicles will be polluted with remains of cortex pieces. This is why the combination with enzymatic isolation might be a better option in the future.

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# 11) APPENDIX

SIGMA-ALDRICH®
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## Product Information

**Percoll®**
Catalog Numbers **P1644, P4937,** and **P7828**

**Percoll® PLUS**
Catalog Numbers **E0414**

Storage Temperature 2–8 °C

**Product Description**
Percoll® is a classic medium for density gradient centrifugation of cells, viruses, and subcellular particles. Percoll consists of colloidal silica particles of 15–30 nm diameter (23% w/w in water), which have been coated with polyvinylpyrrolidone (PVP). The PVP coating renders the product completely non-toxic and ideal for use with biological materials. The PVP is firmly bound to the silica particles as a monomolecular layer. Due to its heterogeneity in particle size, sedimentation occurs at different rates, spontaneously creating very smooth, isometric gradients in the range of 1.0–1.3 g/ml. Most biological particles having sedimentation coefficient values greater than 60S can be successfully isolated in Percoll gradients.

Percoll PLUS is also a silica-based colloidal medium for cell separation by density gradient centrifugation. The silica particles of this medium are covalently coated with silane, providing product stability and long shelf life. The silane coating also provides low osmolality and toxicity, as well as low viscosity. Percoll PLUS has low levels of endotoxins, making it well-suited for cell separation in clinical research applications. Cell separation is performed using the same conditions as the original Percoll. The low toxicity of Percoll and Percoll PLUS ensures that removal of the medium from separated cellular particles is not usually necessary.

Percoll PLUS/Percoll have the following features:

Low osmolality permitting precise adjustment to physiological conditions without significant interference from the medium.

Compatibility with living cells and viruses, allowing separation and recovery of intact, fully active systems.

Impermeable to biological membranes, resulting in no change of buoyant density of particles during centrifugation.

Spontaneous formation of gradient during centrifugation, allowing mixing of large sample volumes in the centrifuge tubes.

Low viscosity resulting in rapid formation of gradients and particle separation.

**Precautions and Disclaimer**
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

**Figure 1.**
Properties of Percoll/Percoll PLUS

| Property | Percoll PLUS | Percoll |
|---|---|---|
| Composition | Silica sol with covalently linked silane | Silica sol with non-dialyzable PVP coating |
| Density (g/ml) | 1.130 ± 0.005 | 1.130 ± 0.005 |
| Osmolality (mOsm/kg $H_2O$) | maximum 30 | maximum 25 |
| Conductivity (mS/m) | – | maximum 100 |
| Viscosity (cP) | maximum 15 (20 °C) | maximum 15 (20 °C) |
| pH | 9.4 ± 0.5 (20 °C) | 9.0 ± 0.5 (20 °C) |
| Endotoxin (EU/ml) | <2 | – |

(Supplier information)

**Preparation Instructions**
Percoll/Percoll PLUS is best used in balanced salt solutions, physiological saline, or 0.25 M sucrose. Cells can be separated in gradients in balanced salts solutions. Subcellular particles, however, tend to aggregate in the presence of salts and it is recommended the separation of such particles be carried out in Percoll/Percoll PLUS diluted with sucrose (0.25 M final concentration).

The low osmolality of Percoll/Percoll PLUS permits this parameter to be controlled by the user without interference from the density medium itself. The addition of 9 parts (v/v) of Percoll/Percoll PLUS to one part (v/v) of either 1.5 M NaCl, 10× concentrated culture medium, or 2.5 M sucrose will result in a solution adjusted to ~340 mOsm/kg $H_2O$. Final adjustments can be made with the addition of salts or distilled water. The precise osmolality should be checked prior to use with an osmometer.

Percoll/Percoll PLUS can be used within the pH range of 5.5–10.0 without any changes in properties. Percoll/Percoll PLUS may form a gel at pH values below 5.5. Gelling can also be caused by the presence of divalent cations, particularly at elevated temperatures.

Percoll/Percoll PLUS will form self-generated gradients by centrifugation at 10,000 × *g* in 0.15 M saline or 25,000 × *g* in 0.25 M sucrose in fixed angle rotors after 15 minutes. Cells or subcellular particles can be mixed with Percoll/Percoll PLUS prior to centrifugation and will band isopycnically as the gradient is formed *in situ*. The use of swinging bucket rotors is not recommended for self-generating gradients.

Percoll/Percoll PLUS may be diluted directly to make a final working solution of known density by the following procedure. In a graduated cylinder, add 1.5 M NaCl or 2.5 M sucrose to 1/10 the desired volume. To this add the required calculated volume of undiluted Percoll/Percoll PLUS (see Figure 2). Make up to the final volume with distilled water.

**Figure 2.**
Volume Calculation

$$V_0 = V \times \frac{\rho - 0.1\rho_{10} - 0.9}{\rho_0 - 1}$$

$V_o$ = Volume of undiluted Percoll/Percoll PLUS required in ml
V = Volume of final working solution in ml
$\rho$ = Desired density of final working solution
$\rho_o$ = Density of Percoll/Percoll PLUS undiluted (lot specific value)
$\rho_{10}$ = Density of 1.5 M NaCl (1.058 g/ml) or 2.5 M sucrose (1.316 g/ml)

The formula is useful for achieving densities that will be very close to the actual densities required. However, slight variations in densities and volumes may affect final density. For highly accurate density requirements, it is recommended to check and adjust the final density using a densitometer or refractometer.The refractive index of diluted Percoll/Percoll PLUS solutions has a linear correlation with the solution density (see Figure 3).

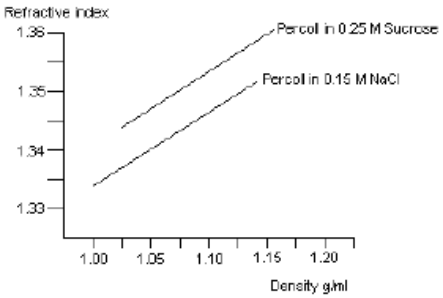


**Figure 3.** Refractive index as a function of density of a Percoll gradient (work from GE Healthcare Bio-Sciences AB, Uppsala Sweden)

**Storage/Stability**
Percoll/Percoll PLUS is aseptically filled and can be stored for up to two years in an unopened container. If stored at –20 °C, gradients form upon thawing, necessitating mixing the contents of the bottle before use.

Preformed gradients can be stored for weeks without a change in gradient shape, provided the gradient is kept under aseptic conditions and not physically disturbed.

Percoll/Percoll PLUS may be autoclaved at 120 °C for 30 minutes without any change in properties. Autoclaving of solutions must be carried out without addition of salts or sucrose. The presence of salts will cause Percoll/Percoll PLUS to gel and the presence of sucrose will cause caramelization.

Minimal contact with air should be maintained during autoclaving to avoid formation of solid particles at the Percoll/Percoll PLUS/air interface. This can be accomplished by using a narrow-top bottle. If particles do form, they may be removed by filtration or low speed centrifugation. If any significant evaporation occurs during autoclaving, the volume should be replenished with sterile water so that the density is not affected. The plastic bottles in which Percoll/Percoll PLUS is packaged are not autoclavable.

**Procedures**

Examples of Separations in Percoll/Percoll PLUS

| Source | Density (g/ml) | Centrifugation Conditions |
|---|---|---|
| Rat Liver Cells | | |
| Hepatocytes | 1.07–1.10 | 30,000 × *g* (30 min) |
| Kupffer cells | 1.05–1.06 | 30,000 × *g* (30 min) |
| Human Cells | | |
| Thrombocytes | 1.04–1.06 | * |
| Lymphocytes | 1.06–1.08 | * |
| Granulocytes | 1.08–1.09 | * |
| Erythrocytes | 1.09–1.10 | * |
| *E. coli* | 1.13 | 30,000 × *g* (20 min) |
| Virus | | |
| Tobacco mosaic | 1.06 | 100,000× *g* (45 min) |
| Equine abortion | 1.08 | 40,000 × *g* (45 min) |
| Influenza | 1.06 | 25,000 × *g* (25 min) |
| Organelles | | |
| Mitochondria | 1.09–1.11 | 50,000 × *g* (45 min) |
| Lysozomes | 1.04–1.07 | 50,000 × *g* (45 min) |
| | 1.08–1.11 | 50,000 × *g* (45 min) |
| Peroxisomes | 1.05–1.07 | 63,000 × *g* (30 min) |
| Synaptosomes | 1.04–1.06 | 50,000 × *g* (45 min) |
| Nuclei | 1.08–1.12 | 100,000 × *g* (60 min) |

Separation of blood cells is best carried out by preforming the gradient (starting density 1.09 g/ml) by centrifugation at 20,000 × *g* for 20 minutes, then layering blood on top of the gradient. Then centrifuge at 1,000 × *g* for 5 minutes in a swinging-bucket rotor, leaving the thrombocytes in the serum layer above the gradient; the serum layer can be removed with a pipette (rate-zonal separation). A further spin for 20 minutes at 1,000 × *g* separates the other cell types at their isopycnic densities.

After centrifugation, the gradient fractions can be collected by puncturing a hole in the bottom of the tube. Another simple and convenient method is to collect the fractions from the tube by displacement with a dense medium such as undiluted Percoll/Percoll PLUS or a 60–65% sucrose solution.

Percoll/Percoll PLUS does not interfere with fluorescent activated cell sorting or with electronic cell counting instruments.

Removal of Percoll/Percoll PLUS from cells

Living cells can be separated from Percoll/Percoll PLUS medium by washing with physiological saline (5 volumes saline to 1 volume of cell suspension). The washing may be repeated two to three times and the cells collected between each washing step by centrifugation at 200 × *g* for 2–10 minutes.

For viruses and subcellular particles, which are too small to be pelleted by low speed centrifugation, the particles can be separated from Percoll/Percoll PLUS by high-speed centrifugation. The undiluted fraction is centrifuged at 100,000 × *g* for two hours in a swinging-bucket rotor or 90 minutes in an angle-head rotor. The biological material remains above the hard pellet of Percoll/Percoll PLUS.

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Information on physical properties and applications was obtained from the supplier.


Lymphocyte Separation

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