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Review article

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

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ABSTRACT

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

1. Introduction

According to recent data, women under 40 years of age have an estimated 2.5% chance of developing cancer (Araujo et al., 2015). However, due to many improvements in cancer diagnosis and treatment (Detti et al., 2012), up to 90% of women who are diagnosed with reproductive tract cancer are now long-term survivors (Jemal et al., 2008). However, as chemo- and radiotherapy often damage ovarian tissue (Rodriguez-Wallberg and Oktay, 2012; Hyman and Tulandi, 2013), patients are likely to show compromised fertility following treatment (Leung et al., 2000; Lee et al., 2006) up to a level where survivors of childhood cancer have an overall reduction of 19% in the likelihood of ever being pregnant (Green et al., 2009). As a consequence, the interest in fertility preservation (FP) strategies in women has been sparked to the extent that it has now become a key medical sub-discipline (Seli and Agarwal, 2012).

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Moreover, fertility preservation is not only a concern in humans, as a parallel need for the preservation of genetic material from endangered animal species or animals with important genetic traits will also greatly benefit from the development of alternative preservation strategies (Comizzoli, 2015). To further optimize FP strategies, the use of animal *in vitro* models will certainly benefit progress in both human and animal FP research (Langbeen et al., 2015a). Recently, there has been an increasing interest for bovine *in vitro* models in human studies on assisted reproductive techniques (ARTs) and FP, as reviewed by Langbeen et al. (2015a). It is well established that a number of similarities exist between bovine and human species with regard to ovarian morphology and function, and that the bovine species could serve as an effective model for human follicular dynamics (Adams and Pierson, 1995; Campbell et al., 2003). Moreover, the easy access to slaughterhouse ovaries guarantees unrestricted availability of study specimens such as PAFs (Langbeen et al., 2015a). Also, 'fertility preservation' in case of the individual female patient will often be translated into 'species preservation' when these techniques are applied to endangered animals, thereby elevating (fertility) preservation from the individual to the population level.

Current FP techniques for women comprise ovarian transposition, cryopreservation of embryos and unfertilized oocytes (Donnez et al., 2006; Wallace, 2011; Revelli et al., 2012) and ovarian tissue cryopreservation (OTC) containing pre-antral follicles (PAFs) (Seli and Agarwal, 2012). In animals, cryopreservation of *in vivo* and *in vitro* produced embryos has become a routinely used technique (Niemann and Wrenzycki, 2018), while freezing of ovarian tissue is still considered to be in its experimental phase. When considering female cancer patients, the most suitable option for a specific patient is based upon different parameters such as the patient's age and relationship status, the type of cancer, and the time available between diagnosis and the onset of treatment (Donnez et al., 2006). The cryopreservation of embryos and oocytes on the other hand requires the patient to be of pubertal age, have a partner or use donor sperm, and to be able to undergo a cycle of ovarian stimulation. For prepubertal girls and women that cannot delay the start of chemotherapy, cryopreservation of ovarian tissue is the only option available. However, this technique is not advisable for patients with certain types of cancer with medium to high risk of ovarian metastasis, such as leukaemia, as there is a risk of re-introducing malignant cells present in the cryopreserved ovarian tissue following auto-transplantation (Rosendahl et al., 2013). For these patients, a safer alternative to allow fertility restoration could be the isolation of pre-antral follicles or PAFs (before or after cryopreservation) from ovarian tissue for *in vitro* growth, maturation, and fertilization or auto-transplantation of the frozen-thawed isolated PAFs in residual ovarian tissue *in situ* (Fisch and Abir, 2018). In animals, (fertility) preservation strategies are often hampered by the urgency that is linked to the procedure. When specimens of endangered animal species die unexpectedly or are found killed under field conditions, the retrieval and cryopreservation of ovarian tissue is often the only rescue option. For animals that already have been dead for a few hours, it might be useful to be able to isolate the PAFs from the surrounding tissue, thereby simultaneously preventing the cryopreservation of ovarian tissue samples that are devoid of follicles. Currently, the main limiting factor is that for many (endangered) species, IVM (*in vitro* maturation) and IVF (*in vitro* fertilization) technologies have not yet been developed. Ovarian tissue xenotransplantation can be applied to maintain the reproductive potential of the donor, but it is still an experimental approach. Using (expensive) rat and mouse immunocompromised hosts, graft rejection has not been a major problem. With regard to species conservation, Snow et al. (2002) harvested mature mouse oocytes from ovarian tissue that was xenotransplanted to a rat recipient that could then subsequently be fertilized and developed into fertile adult mice. Mattiske et al. (2002) observed the development of follicles in grafted cryopreserved ovarian tissue from the tammar wallaby pouch after xenotransplantation into adult mice. Wombat ovarian tissue survived and functioned when grafted into immunocompromised rats (Wolvekamp et al., 2001). Xenografting has the potential to produce mature oocytes from endangered species for use in assisted reproductive technologies, such as *in vitro* fertilization (IVF). Furthermore, mature oocytes from non-endangered species can be used for nuclear transfer for the preservation of critically endangered species.

To encapsulate and protect isolated PAFs, the possibilities of an *in vitro* artificial ovary (Amorim and Shikanov, 2016) and a transplantable artificial ovary are being investigated. An artificial ovary is composed of a matrix that encapsulates and protects not only the isolated follicles but also autologous ovarian cells and bioactive factors, which are necessary for follicle survival and development.

We position the pre-antral ovarian follicle on the forefront for cryopreservation because it plays a major role in ovarian tissue cryobanking and the establishment of alternative storage techniques for isolated follicles. This review will focus on recent developments in (human) fertility preservation strategies which are often accomplished by the use of *in vitro* animal models. It aims to stimulate researchers in the reproductive field to set goals for improving fertility at the individual and preservation at the population level across species.

2. The pre-antral follicle

The follicular reserve stored in the ovaries determines the length of a woman's reproductive life. In domesticated mammals, that are not limited in their reproductive capacities due to menopause, it merely forms a stock from which a cohort of PAFs is activated with each sexual cycle. About 90% of the total follicular reserve, consisting mainly of resting primordial follicles, is stored in the poorly vascularized outer layer of the ovarian cortex. A primordial follicle consists of an immature, quiescent oocyte, surrounded by a single layer of flattened (pre-)granulosa cells. Activation is accompanied by the proliferation and differentiation of granulosa cells: in a primary follicle, granulosa cells are cuboidal in shape and the number of granulosa cells increases. In the subsequent growth stages, the oocyte undergoes volume expansion and a zona pellucida develops (secondary follicle). The majority of activated follicles evolve to the antral stage, characterized by the formation of a cavity or antrum (Aerts and Bols, 2010). Activated follicles migrate towards the more vascularized ovarian medulla (van Wezel and Rodgers, 1996).

Early PAFs account for the vast majority of follicles in the ovarian cortex. The majority of these (99.9%) will never mature into

preovulatory follicles (Morita and Tilly, 1999), but rather will perish at a premature stage along the developmental path. The stock of primordial and primary follicles thus represents an untapped potential, which could be cultivated for reproduction, preservation, or research purposes. Improvements in the isolation, *in vitro* culture and auto-transplantation of ovarian follicles will therefore greatly enhance current fertility preservation strategies. They provide a safe method for the restoration of fertility in women that are affected by chemo/radiotherapy induced premature ovarian failure. Additionally, they also constitute a genetic pool from which endangered animal populations can be restored when *in vitro* follicle culture will become a mainstream technique.

3. Options for fertility preservation

3.1. Ovarian tissue

Although several dozens of births have already been reported worldwide (Donnez et al., 2011, 2012; Jadoul et al., 2017), auto-transplantation of frozen-thawed tissue cannot be recommended to cancer patients who are at risk of the reintroduction of malignant cells (Dolmans et al., 2010; Rosendahl et al., 2010; Van Eyck et al., 2010). Dolmans et al. (2010) evaluated the presence of leukemic cells in cryopreserved ovarian tissue from leukemia patients. They found that histological analyses did not identify any malignant cells in the tissue, however, quantitative reverse-transcribed polymerase chain reaction (RT-PCR), demonstrated ovarian contamination by malignant cells in acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML). Immunodeficient mice xenografted for 6 months with ovarian tissue from ALL patients, developed intraperitoneal leukemic masses, demonstrating that reimplantation of cryopreserved ovarian tissue from patients with ALL puts them at risk of disease recurrence.

Another drawback linked to transplantation of ovarian tissue is the extensive loss of PAFs due to delayed and deficient re-vascularization of the transplanted tissue (Martinez-Madrid et al., 2009; Van Eyck et al., 2010). From only about 500 to 1,000 primordial follicles present in the transplanted ovarian tissue strip, more than 50% are lost (Donnez et al., 2004). Several attempts have been made to treat or prepare tissue strips, aiming for a better vascularization following transplantation, such as co-incubation with vascular endothelial growth factor (VEGF) (Langbeen et al., 2016). *In vitro* VEGF exposure of bovine cortical tissue strips prior to transplantation in immunodeficient mice was shown to be beneficial only for short term survival of PAFs, 2 weeks following transplantation (Fig. 1). However, 4 weeks after transplantation, no additional beneficial effects of VEGF on the vascularization of the grafted ovarian tissue could be reported. It was concluded that, although the transplantation process overall negatively influences the number of viable follicles and vascular density, VEGF exposure prior to transplantation can favor follicle survival during a 2 weeks transplantation period.

While chances of restoration of ovarian activity is higher if primordial follicles are present in the transplanted tissue and the chance of live birth after replacement of ovarian tissue is approximately 20% (Donnez et al., 2013), both quantitative and qualitative assessments of the follicle population in ovarian tissue samples are difficult, if not impossible (Shaw et al., 2000a; Amorim et al., 2003a; Jorssen et al., 2014). Indeed, PAFs are heterogeneously distributed and clustered throughout the ovarian cortex. Every developmental stage can be present in any given piece of ovarian cortex and many pieces might contain no follicles at all. As a consequence, the risk of freezing tissue samples devoid of (viable) follicles is significant, creating a false idea of preserved fertility for the patient.

Although ovarian tissue cryopreservation (OTC) is still considered to be in its experimental phase, many experts believe that there is now sufficient evidence to consider it as a valid and effective technique (Donnez and Dolmans, 2015, 2017). However, because laparoscopic oophorectomy and ovarian cortex collection is an invasive procedure requiring general anesthesia, thorough patient selection is essential (Wallace et al., 2016) with age being one of the most important selection criteria. While first-line cancer treatment does not compromise the ovarian reserve (= the amount of PAFs present in a quiescent state on both ovaries) by more than 10% in girls under 10 years of age (Wallace et al., 2014, 2016), girls at the age of 11 or 12 years have an estimated 30% decline in their ovarian reserve. Further selection criteria are a realistic chance of survival for 5 years together with a high risk of premature

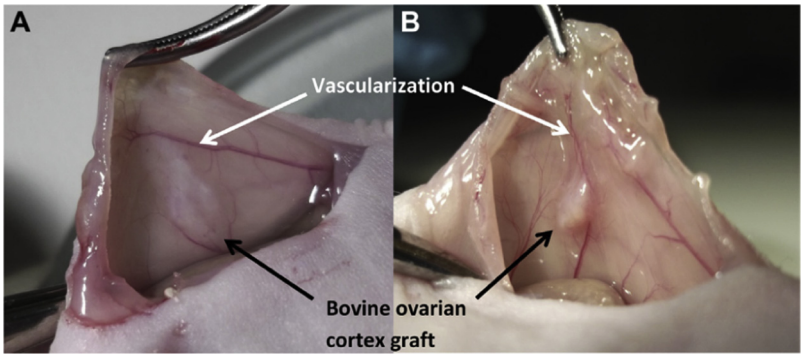

Fig. 1. Transfer of bovine ovarian cortex as a relevant model for human (preimplantation) reproductive research at a retroperitoneal location in an immune deficient mouse serving as an *in vivo* bioincubator. Macroscopic graft aspect after a transplantation period of 2 weeks (A) and 4 weeks (B), with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

ovarian insufficiency (> 50%) (Wallace et al., 2014, 2016). Finally, in human, there seems to be a correlation between the intensity of cancer therapy and the likelihood of premature ovarian insufficiency, even in young girls (Salih et al., 2015; El Issaoui et al., 2016). Unfortunately, it is still impossible to predict the effect of aggressive chemotherapy on the ovarian reserve.

Due to ethical issues and the scarcity of research material, it is problematic to perform experiments using human tissue. Thus, advances in bovine and sheep ovarian vitrification are relevant, as these have been demonstrated to be anatomically and physiologically similar to the human ovary (Kagawa et al., 2009; Lunardi et al., 2015).

3.2. Isolated follicles

PAFs are surrounded by a basement membrane that separates them from the ovarian stroma, blood vessels, white blood cells and innervation (Rodgers et al., 2003). Therefore, malignant cells cannot invade PAFs via the blood stream and as a consequence, cannot be returned back to the patient when isolated PAFs would be auto-transplanted in residual ovarian tissue following cancer treatment. In human reproductive research, two main strategies are being investigated worldwide to allow fertility restoration and to avoid the risk of transplanting malignant cells: the *in vitro* artificial ovary and the 'transplantable' artificial ovary (Amorim and Shikanov, 2016). While the first one aims to achieve the entire folliculogenesis process *ex vivo/ in vitro*, the second aims to transplant isolated pre-antral follicles back to their natural environment, albeit in an artificial scaffold.

Primordial follicles are considered to be more resistant to cryo-injury than growing follicles because they only have a small amount of cold sensitive intra-cytoplasmatic lipid droplets, are less differentiated and their metabolism is relatively low, they only have a small amount of organelles and lack a zona pellucida, cortical granules and a meiotic spindle (Hovatta et al., 1996; Shaw et al., 2000b).

The small size of primordial follicles (between 30 and 80 μm) also greatly facilitates penetration of cryoprotectants (Shaw et al., 2000b). Because these PAFs have been shown to easily maintain normal morphology and ultrastructure during cryopreservation, they are excellent candidates for long term preservation.

When it comes to PAF isolation, both mechanical and enzymatic methods (Figueiredo et al., 1993; Dolmans et al., 2006) are used to separate follicles from the surrounding stromal cells. Enzymatic digestion of small ovarian tissue fragments is reasonably fast, but it might damage the basal membrane, impairing the follicle's viability (Figueiredo et al., 1993). Mechanical follicle isolation, on the other hand, is generally considered to be a safe but labour-intensive and time-consuming method (Figueiredo et al., 1993). For human primordial follicles, however, mechanical isolation is difficult due to the fibrous and dense ovarian stroma, and therefore enzymatic digestion with collagenase or liberase has to be used (Dolmans et al., 2006). The originally developed mechanical procedure for isolating PAFs (as used for ruminant ovaries) consists of cutting and pipetting followed by filtration, and yields a large number of follicles (Figueiredo et al., 1993). More recently, Langbeen et al. (2015b) developed a new mechanical isolation method based on tissue dispersion following cutting and blending small tissue pieces of ovarian cortex. Applied to the ovaries of three ruminant species, this consistently results in the retrieval of viable follicles without visual damage to the basement membrane. In addition, the mechanically isolated follicles can easily be characterized and classified upon retrieval based on generally accepted morphological characteristics, such as follicular diameter and the number of granulosa cells (Braw-Tal and Yossefi, 1997; Rodgers and Irving-Rodgers, 2010). In animals, ovarian tissue can quite easily be collected post-mortem, and PAF retrieval is also possible using the transvaginal, ultrasound-guided biopsy collection approach (Aerts et al., 2005). Thus avoiding a laparoscopic intervention, it is possible to repeatedly sample the ovary and collect small pieces of ovarian cortex tissue containing viable PAFs from living donor cows (Aerts et al., 2008a). This method can eventually be developed into a new source of PAFs for *in vitro* maturation of follicles for high genetic merit livestock production as soon as routine *in vitro* follicle culture protocols will be available.

4. Cryopreservation

4.1. Ovarian tissue

Human reproductive researchers reported the first successful pregnancy and childbirth following the auto-transplantation of strips of cryopreserved ovarian cortex in 2004 (Donnez et al. (2004). Thus far, more than 60 live births have been achieved after re-implantation of cryopreserved ovarian tissue (Donnez and Dolmans, 2015). Most reported live human births were achieved after transplantation of ovarian tissue that had been slow-frozen (Donnez et al., 2013), a technique that is still applied in most FP laboratories and based on a computer-assisted gradual and well controlled cooling down protocol. There are only a few reports of live births from transplantation of vitrified human ovarian tissue (Kawamura et al., 2013; Suzuki et al., 2015). Although rapid advances in vitrification methods - basically avoiding the formation of ice crystals by ultra-rapid cooling and the use of high concentrations of cryoprotectants - have led to successful cryopreservation of embryos and mature oocytes, studies comparing slow freezing and vitrification of ovarian tissue have presented conflicting results (Kim et al., 2015b).

The vitrification of ovarian tissue is difficult because of cellular heterogeneity. The permeation and toxicity of cryoprotectants (CPAs) are specific for each cell type and tissue. The most effective combination and concentration of the CPAs used is therefore a compromise between optimal values for the different cell types (oocyte, granulosa cells, stromal cells, blood vessels). The surrounding ovarian tissue may result in a slower and more complex perfusion process, potentially leading to reduced oocyte protection during all steps of the cryopreservation procedure. Solid surface vitrification (SSV) uses a metal cube covered with aluminum foil which is pre-cooled to −180 °C by partial submersion in liquid nitrogen (LN2). Microdrops of vitrification solution, containing the samples, are dropped onto the cold upper surface of the metal cube and are instantaneously vitrified (Dinnyés et al., 2000). SSV provides sufficient

space for the tissue, maximizes cooling rates, and avoids the generation of the gas phase of LN2 bubbles and has been successfully applied to both goat and sheep ovarian tissues (Santos et al., 2007a; Lunardi et al., 2015) as well as goat, bovine, porcine and sheep oocytes (Begin et al., 2003; Somfai et al., 2007; Sripunya et al., 2009; Lunardi et al., 2015). Kagawa et al. (2007) reported the birth of healthy mouse pups derived from the oocytes of pre-antral follicles obtained from ovarian tissue of adult mice that were vitrified using the Cryotop vitrification technique. The Cryotop method was designed by Kuwayama and consists of a fine, transparent polypropylene film attached to a plastic handle equipped with a cover straw, into which oocytes and embryos can be loaded in very small volumes (˜0.1 μl) (Kuwayama, 2007). Based on this high-efficiency vitrification Cryotop method, a Cryotissue method was developed for the vitrification of ovarian tissue. Bovine and human ovarian tissue have been successfully vitrified using the Cryotissue method. Ovarian tissue was placed in a minimum volume of solution onto a thin metal strip (Cryotissue; Kitazato BioPharma, Fujinomiya, Japan), and submerged directly into sterile LN2, and subsequently the strip was inserted into a protective container and placed into an LN2 storage tank. No difference in oocyte viability (> 89%) between fresh and vitrified ovarian cortical tissue in either bovine or human samples was found. Autotransplantation of vitrified–warmed tissue back to cattle-donors also resulted in no loss of oocyte viability (Kagawa et al., 2009).According to some human studies, slow freezing yielded better results in cryopreserving ovarian tissue (Isachenko et al., 2009, 2010), while others found improved conservation of the ovarian follicular and stromal structures and increased follicle survival rates after vitrification (Kagawa et al., 2009; Keros et al., 2009; Amorim et al., 2011; Herraiz et al., 2014; Dalman et al., 2017). With an increase in the number of children born, data on the efficiency of vitrification and slow freezing of ovarian cortex can be compared and validated in prospective randomised studies, with healthy livebirth rates as the main outcome parameter.

Other researchers (Aerts et al., 2008b) reported the survival and growth of auto-transplanted murine follicles after cryopreservation of the ovarian cortex by two different methodologies, solid-surface vitrification (SSV) later also known as Cryologic vitrification method (CVM) and slow-rate freezing (SRF).

CVM vitrified ovarian fragments were loaded in 3 μL droplets onto the fine hook at the end of a plastic fibreplug, after which the droplets were vitrified by contact with the metal surface. Slow-rate frozen ovarian fragments were cooled using a programmable freezer and plunged in LN2. Increased follicular proliferation and antral stages were identified after both cryo-treatments. The fraction of secondary and antral follicles was, however, significantly larger after SRF cryo-treatment, suggesting that CVM treated tissue may have suffered a growth disadvantage. Vitrification of pre-antral follicles by the CVM method remains a viable alternative to conventional SRF, but further research is required for optimizing and standardizing ovarian tissue cryopreservation, with a special focus on follicle quality and viability assessment, which is difficult for follicles *'in situ'*.

4.2. Isolated follicles

Results obtained from studies on the cryopreservation of isolated PAFs have been encouraging. Thus far, studies on slow freezing have been performed in sheep (Amorim et al., 2003b, 2004; Amorim et al., 2006; Santos et al., 2007b), goat (Rodrigues et al., 2005), cat (Jewgenow et al., 1998), monkey (Barrett et al., 2010) and human isolated follicles (Vanacker et al., 2013). It was demonstrated in a study by Vanacker et al. (2013) that human PAFs can be successfully cryopreserved by slow freezing before or after isolation, without impairing their ability to survive and grow *in vitro*.

Vitrification of isolated follicles was investigated in mice (Segino et al., 2003), rat (Xing et al., 2010), sheep (Amorim et al., 2004; Lunardi et al., 2015) and cattle (Langbeen et al., 2014). Lunardi et al. (2015) vitrified sheep secondary follicles in the isolated state and within fragments of ovarian tissue. They concluded that both techniques can be used, but isolated follicles displayed a better follicular growth rate and in this group, fewer follicles with a decreased diameter were found after *in vitro* culture.

Isolated bovine follicles were successfully vitrified using HSV straws®, showing a high viability (87.5%) post thawing with no significant differences to follicles that were cultured in a 2D culture system (Bus et al., 2018). However, considering the labor-intensive procedure and relatively low efficiency as many PAFs are lost during the vitrification procedure, vitrification of embedded follicles, in for example alginate beads (see below) may be the method of choice in the future. Langbeen et al. (2014) demonstrated that isolated PAFs are much easier to quantify and assess for viability, which is a crucial component of a functional fertility preservation strategy (see below).

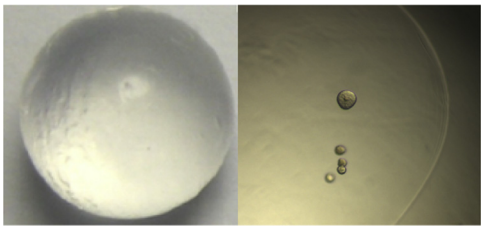

Fig. 2. Left: macroscopic image of an alginate bead. Right: light microscopic image of 5 encapsulated follicles in an alginate bead (Figure Bus et al., 2018).

4.3. Isolated embedded follicles

Bus et al. (2018) studied the effects of encapsulation of bovine isolated PAFs in calcium alginate beads on follicle viability and morphology after vitrification (Fig. 2). Follicles that were vitrified in alginate beads carried in mesh cups showed a low viability post warming (45.9%) and were significantly less viable than non-vitrified PAFs that were cultured directly in beads (88.4%). However, the encapsulation of follicles in beads has important advantages, as their manipulation is much easier and a lot of time is saved because follicles can be vitrified in small groups. While vitrification as such was successfully used for non-embedded PAFs, further optimization for embedded bovine PAFs is necessary and seems advantageous. A first step in this process could be the use of a longer exposure time to the cryoprotectant agents (CPAs), considering the increased volume of embedded follicles which may prolong the CPA diffusion time (Bian et al., 2013) (Sadeghnia et al., 2016). In addition, Bian et al. (2013) showed that human PAFs encapsulated in alginate could maintain their normal ultrastructure after vitrification. Camboni et al. (2013) successfully cryopreserved embedded human primordial/primary follicles using slow freezing. In conclusion, alginate constitutes an easy-to-handle, safe hydrogel matrix to cryopreserve isolated follicles, but further studies are necessary to increase the survival and maintenance of normal morphology after vitrification.

5. Follicle quality assessment

5.1. Short term in vitro culture

Survival and follicle growth during short term *in vitro* culture can be used as a non-invasive tool for the evaluation of follicle *in vitro* viability. To generate individual follicle follow-up data, Jorssen et al. (2015) characterized follicular dynamics using an *in vitro* culture system of isolated and individually cultured bovine early pre-antral follicles during 10 days. Individual follicle morphology and growth were evaluated by non-invasive assessment methods that allowed continuous evaluation over time. PAFs were light microscopically evaluated during culture to assess macroscopic cell morphology and follicle diameter. Based on the connection between the oocyte and the surrounding (pre-)granulosa cells and the microscopical integrity of the basal membrane, follicles were subdivided into three categories, with category 1 follicles showing the best morphological features (Fig. 3). As shown with Neutral Red staining (see below), nearly 68% of all PAFs (cat. 1, 2 and 3) survived a 10-day *in vitro* culture period, whereby an intact basal membrane and connection between the oocyte and the granulosa cells seem to be a prerequisite for maintaining follicular development. The increase in follicular diameter was found to be correlated with an increase in the total cell number. Follicles maintaining their category 1 morphologic features over time seemed to be of a better quality and showed a higher developmental competence *in vitro* as compared to category 2 and 3 follicles (Jorssen et al., 2015).

5.2. Neutral red staining

Neutral Red (NR) is a water-soluble and nontoxic dye that was proposed as a non-invasive viability assay on the basis of its ability to diffuse through the plasma membrane and concentrate in the lysosomes of viable and metabolically active cells (Elliott and Auersperg, 1993; Repetto et al., 2008). It can be used to evaluate follicle content and viability in cortical tissue strips or for the staining of isolated follicles (Fig. 4) (Chambers et al., 2010). Kristensen et al. (2010) validated the use of NR as a vital dye through the application of the carboxyfluorescein diacetate succinimidyl ester as a marker for proliferation after the use of NR, therefore suggesting the absence of (acute) NR toxicity. Langbeen et al. (2014) established a staining protocol for isolated bovine PAFs, indicating that 15 mg/mL of NR, with an exposure time of 30 min, was not deleterious when used before and after vitrification. In addition, Jorssen et al. (2014) reported that NR could be used to identify PAFs present in bovine cortical tissue strips and concluded that *in vitro* follicular dynamics were not influenced by oxygen tension or by repeated viability assessments using NR.

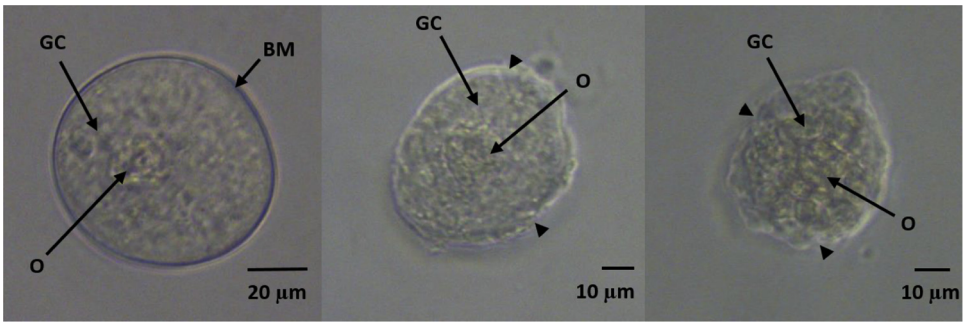

Fig. 3. A) Category 1 includes the follicles with both an intact basal membrane and an intact connection between the oocyte and surrounding granulosa cells. B) Category 2 contains follicles with an intact connection between the oocyte and the surrounding granulosa cells but shows signs of a disrupted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

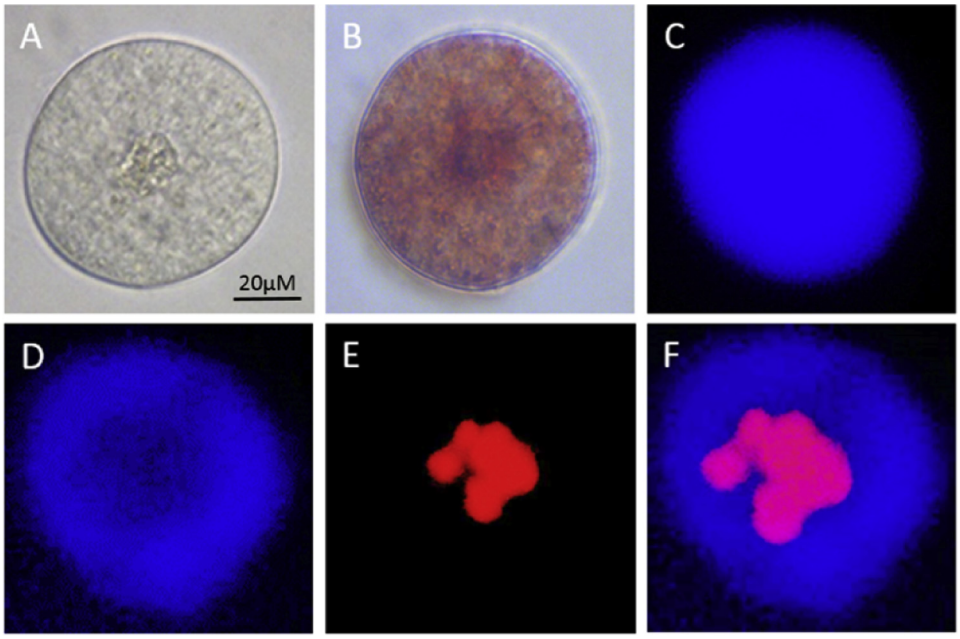

Fig. 4. Follicles stained with Neutral Red (NR) and calcein Blue AM to indicate the viability of the follicles, propidium iodide shows nuclei of dead cells. A) Light microscopic (LM) image of non-stained follicle. B) LM image of follicle staining positive for NR; viable follicle. C) Follicle staining positive for calcein; viable follicle. D) Follicle of which the oocyte stained negative and the granulosa cells positive for calcein. E) Same follicle as in B, nuclei staining positive for propidium iodide. F) Merged picture of D and E (Figure Bus et al., 2018) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

5.3. Calcein staining

Follicle viability can easily be assessed with the fluorescent probe Calcein (Blue) AM. Through intracellular esterase activity, living cells convert the non-fluorescent cell-permeable Calcein-AM into a fluorescent calcein. The polyanionic dye calcein is retained well in living cells, producing an intense, uniform green or blue (Calcein Blue AM) fluorescence. Green fluorescence can be visualized after exposing the tissue to light with a wavelength of 495 nm (excitation [ex]) and observing the emitted (em) light at a wavelength of 515 nm. Blue fluorescence can be visualized with excitation/emission maxima of 360/449 nm. Staining with Calcein (Blue) AM permits follicle viability to be assessed within 1 h (Cortvrindt and Smitz, 2001).

5.4. Gap junction identification and assessment of functionality

Gap junctions are intercellular membrane channels directly connecting the cytoplasm of adjacent cells, thus allowing the exchange of ions, second messengers and small metabolites (Larsen, 1983), and they are thought to play a crucial role in intercellular communication between different follicular components (Grazul-Bilska et al., 1997). A gap junction channel is composed of two hemi-channels (connexons), each of which is composed of six protein subunits (connexins). Among gap junction proteins identified in ovarian follicular cells, the two connexins (Cx) Cx37 and Cx43 seem to be critical at each step of normal folliculogenesis (Kidder and Vanderhyden, 2010). Investigating Cx expression in the bovine ovary as a prerequisite for follicle quality, Nuttinck et al. (2000) found that Cx43 expression was restricted to granulosa cells, while Cx37 staining was observed in both the oocyte and granulosa cell compartments. Vitrification of follicles can lead to cryoinjuries, which can result in the loss of membrane proteins, such as Cx37 and Cx43. Ortiz-Escribano et al. (2017) showed that vitrification opens hemichannels in bovine blastocysts, which are normally closed, but open in response to stress conditions and concluded that blocking hemichannels can protect embryos during vitrification and warming. After cryopreservation and subcutaneous transplantation of mouse ovarian tissue, proteins forming gap junctions between oocytes and granulosa cells are under-expressed compared with normal controls (Lee et al., 2008). The poor retrieval of mature oocytes from such grafts might in part result from the failure of signal transduction and metabolite transmission between granulosa cells and the oocyte. In isolated PAFs, the detection and quantification of immunofluorescently labeled connexins before and after cryopreservation can give an indication about the functional state of follicles post thawing. Functionality of gap junctions can be assessed by injection of the fluorescent dye Lucifer Yellow into the oocyte and by examination of the dye's ability to transfer from the oocyte to the surrounding granulosa cells through intact gap junctions. Barrett et al. (2010) showed that immediately after thawing, mouse follicles were unable to transfer dye into surrounding granulosa cells, indicating that the gap junctions were not functional. However, after 2 days of culture, cryopreserved follicles were able to re-establish gap junctions and transport the dye from the oocyte into the surrounding somatic cells.

5.5. Xenotransplantation

Xenotransplantation of ovarian tissue or isolated PAFs can be used to assess the viability and developmental capacity of PAFs. Massive follicle activation is a typical transplantation effect, but testifies to the survival of cryopreserved follicles (Aerts et al., 2008b). In the study by Aerts et al. (2008b), the fraction of primordial follicles in the Cryologic vitrification method (CVM) and slow-rate freezing (SRF) grafts significantly decreased as compared to control tissue, whereas intermediary and primary follicles significantly increased, indicating massive follicle activation following transplantation. Such massive primordial follicle recruitment typically occurs after grafting, but also during *in vitro* culture experiments. It is hypothesized that the ovary under physiological conditions is dominated by inhibitory mechanisms (Baird et al., 1999), which are no longer available during culture or transplantation. An alternative explanation was proposed by Cushman et al. (2002) who suggested that the follicle environment *in vitro* or after grafting was richer in oxygen and nutrient levels than the avascular ovarian cortex, which contains the quiescent primordial follicles.

6. Post-thawing applications with isolated follicles

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

Culturing isolated follicles *in vitro* from the primordial stage onwards is potentially an attractive strategy because they represent > 90% of the total follicular reserve and show high cryotolerance (Smitz and Cortvrindt, 2002). However, to date, it has not been possible to grow human and large domestic mammal isolated primordial follicles *in vitro* to the mature oocyte stage (Abir et al., 2001; Torrents et al., 2003). Therefore, further studies are needed to identify factors sustaining follicular growth and maturation and to assess the contribution of stromal cells to these processes. Primary and early secondary follicles are gonadotropin independent and essential factors for their growth and development often require co-culture with various “feeder” cells/follicles (Green and Shikanov, 2016). Beyond the two-layer stage, follicle stimulating hormone (FSH) is required for further growth (Oktay et al., 1998).

Isolated single follicles can be cultured in two-dimensional (2D) systems (Wu et al., 2001; Jorssen et al., 2015), in three-dimensional (3D) systems (Pangas et al., 2003), or in a multistep system (Picton et al., 2008; Telfer and McLaughlin, 2011). In conventional 2D culture systems, follicles tend to flatten on the bottom of the culture plate, inducing breakdown of the surrounding basal lamina, causing granulosa cells to detach from each other and the oocyte. Follicular flattening interferes with the essential bi-directional communication between oocyte and granulosa cells via gap junctions, which makes it unable to complete maturation. Bovine follicles in 2D culture successfully produced estradiol and formed antral cavities, suggesting that 2D culture is capable of supporting follicular viability and function, but only to a certain level (McLaughlin et al., 2010).

The *in vivo* development from the primordial to preovulatory follicle stage is a time-consuming event, estimated to be 180 days in the cow (Lussier et al., 1987), 205 days in humans (Gougeon, 1996) and 20 days in mice (Pedersen, 1970). To attain successful *in vitro* follicular growth, more research is necessary to optimize each progressive step in follicular maturation, to more closely mimic the *in vivo* environment.

A multi-step *in vitro* culture system has been designed and this is thought to be the only effective method for obtaining good quality oocytes for successful fertilization in humans, non-human primates and large animals (Picton et al., 2008; Telfer and McLaughlin, 2011). In this approach, the activation of follicle growth is promoted within fragments of the ovarian cortex and followed by mechanical isolation and individual culture of secondary follicles within a 3D culture system (*e.g.* alginate hydrogel, see below) (McLaughlin and Telfer, 2010), as the cortical environment becomes inhibitory for growth to the antral stage. Following formation of the antrum, the oocyte within can also be mechanically extracted from the *in vitro* matured follicle, and subsequently undergo *in vitro* fertilization (Smitz et al., 2010; Telfer and McLaughlin, 2011).Sadr et al. (2015) found that 3D culture systems are more appropriate than a 2D approach with regard to maintaining the spherical morphology, growth rate, and gene expression patterns associated with normal mouse oocyte development. In 3D culture systems, follicles are embedded in a matrix, maintaining the spherical morphology of the follicle and preserving the cell-cell and cell-matrix interactions within the stromal tissue. This allows the follicle to successfully complete maturation. The composition of the used biomaterials mimic the natural environment to meet the physiologic needs of the follicles. Substances used should be non-cytotoxic and need to have a certain elasticity to allow expansion of the granulosa cells. In addition, these matrices need to allow adequate gas exchange, diffusion of nutrients and removal of cellular waste. Alginate hydrogels are currently the most popular material to embed and culture isolated PAFs (Green and Shikanov, 2016). In mice, live offspring were reported from follicles cultured using calcium alginate (Xu et al., 2006). Human secondary follicles embedded in calcium alginate gels maintain their spherical structure, survive for up to 30 days, develop into small antral follicles and preserve their zonal projections (Xu et al., 2009; Yin et al., 2016). The physical characteristics of the 3D-matrix are important to consider, as stiffness and pore size must be carefully adapted to the species and the follicle stage being cultured. As the ovarian stroma of humans and primates is more rigid than other species, especially in early stage follicles, they require a stiffer biomaterial to optimize *in vitro* growth. Murine and caprine models require a less rigid, more flexible scaffold for optimized follicle growth. The combination of alginate matrix with fibrin creates a dynamic environment in which during follicle growth follicular proteases degrade fibrin, decreasing the rigidity of the matrix (Shikanov et al., 2011).

6.2. Transplantable artificial ovary

Recently, considerable efforts have been made in the development of an artificial ovary for transplantation, which encompasses a matrix that encapsulates, protects and maintains the 3D structure of follicles and mimics the natural environment of the ovary. The

matrix should not only encapsulate cryopreserved-thawed follicles but also autologous ovarian cells and bioactive factors, which are necessary for follicle survival and development (Dolmans et al., 2007; Amorim, 2016). A second biopsy after cancer remission is needed to harvest the required autologous ovarian cells (OCs). This avoids the risk of adding malignant cells to the artificial ovary (Amorim, 2016). Moreover, cryopreservation proved harmful to OCs, decreasing their number and viability, while chemotherapy does not seem to have a negative impact on OCs (Soares et al., 2015). The artificial ovary would potentially restore both fertility and endocrine function once transplanted into the patient (Vanacker et al., 2012a). The artificial ovary would also be a unique research tool for investigating folliculogenesis, a complex process that is far from being fully understood. Basic features required for an artificial ovary are its non-cytotoxicity, being biodegradable and biocompatible and being able to encapsulate cells, which makes the identification of an optimal matrix a key challenge. Several polymers have been tested for use as an artificial ovary matrix. One of the first studies in the development of the artificial ovary was performed by Gosden (1990). In this study, pups were obtained after allotransplantation of isolated murine follicles and OCs encapsulated in autologous plasma clots. It was also demonstrated that murine follicles isolated from cryopreserved ovaries encapsulated in plasma clots were able to yield offspring (Carroll and Gosden, 1993). Plasma clots were also used to transplant human isolated pre-antral follicles to the ovarian bursa of immunodeficient mice. This resulted in the development of secondary follicles (Dolmans et al., 2007) and, after five months antral follicles (Dolmans et al., 2008). However, plasma clots have an inconsistent composition and degrade quickly, which can lead to follicle loss and variable results.

Vanacker et al. (Vanacker et al., 2012b, 2014) were the first to perform studies with alginate as a matrix for a transplantable ovary. In a first study, they autografted successfully isolated mouse ovarian cells encapsulated in a matrix of alginate and matrigel into a pocket in the internal part of the mouse peritoneum. After one week, the matrix was able to degrade, allowed vascularization, and supported cell survival and proliferation and elicited only a moderate inflammatory response (Vanacker et al., 2012b). In a second study, isolated mouse pre-antral follicles and OCs were grafted in a 1% SLM alginate matrix to a peritoneal pocket for one week. The authors recovered 22% of grafted follicles, including antral stage specimens. The alginate matrix was invaded by proliferating cells and vessels were formed, but the degradation rate was slow and vascularization was observed mainly at the periphery of the graft (Vanacker et al., 2014). These studies demonstrate that alginate is a promising candidate for the creation of an artificial ovary.

Fibrin is a natural polymer and its physical properties can easily be modulated by varying fibrinogen and thrombin concentrations (Amorim and Shikanov, 2016). Pups were obtained after the transplantation of murine isolated pre-antral follicles to the ovarian bursa when encapsulated in fibrin-VEGF clots (Kniazeva et al., 2015). Synthetic polymers can be produced in large uniform quantities and can be combined with bioactive compounds to stimulate cellular adhesion, proliferation and differentiation (Yoon and Fisher, 2009). Poly-etheleneglycol (PEG) was investigated by Kim et al. (2015a), whom encapsulated isolated primordial murine follicles to PEG modified with vinyl sulfone groups, and transplanted them to the ovarian bursa of mice for 30 days, after which antral follicles were found. To overcome the problems associated with the matrix, such as slow vascularization, Aerts et al. (2010) tested xenotransplantation devoid of solid vehicle support, in order to improve follicle survival and growth. A suspension of isolated pre-antral follicles and stroma cells were micro-injected under the kidney capsule of nude mice (Fig. 5). A massive follicular activation was seen after 14 days of transplantation, indicating that isolated pre-antral follicles were able to survive and grow.

7. Conclusions

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

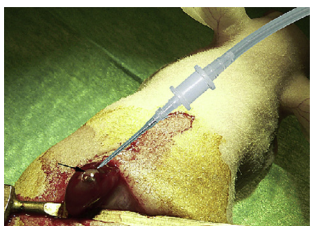

Fig. 5. Xenotransplantation of isolated pre-antral follicles to the kidney capsule of a nude mouse. The pipette tip was introduced through a capsulotomy to deposit the follicular suspension (arrow) (Figure Aerts et al., 2010).

Conflict of interest

The authors declare that there is no conflict of interest in publishing this review. All (co-)authors state that the funding of this research is provided by the independent Operational Costs of the University of Antwerp.

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