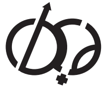


Anim. Reprod., v.10, n.3, p.187-198, Jul./Sept. 2013

Equine preantral follicle harvesting, processing, and in vitro culture: the journey has already started

K.T. Haag[1], D.M. Magalhães-Padilha[1,2], M.O. Gastal[1], J.R. Figueiredo[2], E.L. Gastal[1,3]

[1]Department of Animal Science, Food and Nutrition, Southern Illinois University, Carbondale, IL, USA.
[2]Faculty of Veterinary Medicine, State University of Ceará, Fortaleza, CE, Brazil.

Abstract

Preantral follicles are of great abundance in mammalian ovaries and the vast majority (>99.9%) never become ovulatory; therefore, the ability to rescue these otherwise wasted follicles seems very appealing. Considering there are striking similarities in antral follicle dynamics between mares and women, the mare might become a good model to study early (preantral and antral) folliculogenesis in women, with several advantages related to using an animal model. Studies in our laboratory recently validated the use of a transvaginal, ultrasound-guided ovarian Biopsy Pick-Up (BPU) method to harvest preantral follicles using the mare as a model to study early folliculogenesis (Haag *et al*., 2013a, b, c). This article will review some of the important findings of our recent studies related to the harvesting, processing, and culture of equine preantral follicles and discuss those with the limited information available in the literature.

Keywords: biopsy pick-up, equine, folliculogenesis, ovary, preantral follicle.

Introduction

The importance of research involving preantral follicles has become more apparent in recent decades. Oocytes are in great abundance in the mammalian ovary, yet only a very small portion (<0.01%) of these oocytes are ever released from preovulatory follicles via ovulation and have a chance to be potentially fertilized. A vast majority of oocytes are enclosed within preantral follicles which will either die by the process of follicular atresia at some point during development or never become activated and remain dormant. Therefore, the ability to rescue these otherwise wasted follicles is very attractive.

The development of technologies to harvest and isolate preantral follicles and mature them *in vitro* holds many promising applications because of the great abundance of preantral follicles in the mammalian ovary. Cryopreservation and/or *in vitro* culture of preantral follicles could potentially serve many purposes, such as large-scale embryo production from individuals with high genetic merit, establishment of gamete banks for rare or endangered species, advancement of knowledge for contraception purposes in wild animals, development of bioassays to test toxic effects of pharmaceutical and environmental agents, and preservation of fertility in humans whose preantral follicle population has been jeopardized by chemotherapy and/or radiotherapy cancer treatments, which partially or entirely eliminate the follicle reserve (Picton *et al.*, 2000). The success of these technologies is completely dependent upon understanding the specific mechanisms that regulate follicle and oocyte growth and development. The amazing benefits that could potentially be provided through *in vitro* culture of preantral follicles make the understanding of early folliculogenesis a top priority research area. During the past 10 yr, the field of ovarian folliculogenesis has seen a large amount of focus placed upon the study of preantral follicles. Although there is limited knowledge of the mechanisms that control preantral follicle dynamics, researchers are beginning to understand how preantral follicles undergo activation and growth through *in vitro* culture studies.

In light of the fact that there are several noteworthy similarities between women and mares regarding antral follicle dynamics (Ginther *et al*., 2004, 2005; Baerwald, 2009; Gastal, 2009, 2011; Ginther, 2012), the mare could potentially become an appropriate model for studying early folliculogenesis in women, with an animal model being advantageous in numerous ways. Obtaining material to be used for *in vitro* culture can be difficult, mainly because for several species the only sources of preantral follicles are slaughterhouse or ovariectomized ovaries. This problem is even more amplified in mares, especially in the United States where accessible ovarian tissue is very scarce due to the closing of all equine abattoirs in 2007. Hence, a transvaginal, ultrasound-guided ovarian biopsy procedure that would allow for the repeated collection of preantral follicles *in vivo* could be very beneficial in providing material for the study of early ovarian folliculogenesis *in vitro* (Lass *et al*., 1997; Aerts *et al*., 2005).

In order to expand upon the limited understanding of early folliculogenesis in the equine species, the following hypotheses were tested in our recent studies (Haag *et al*., 2013a, b, c): 1) the transvaginal, ultrasound-guided Biopsy Pick-Up (BPU) method provides sufficient material for studies on the early stages (primordial, transitional, and primary follicles; Fig. 1) of folliculogenesis in mares; 2) preantral follicle quantity, viability, and morphology do not differ according to phase of the estrous cycle; 3) younger mares have more follicles per mg of tissue than

[3]Corresponding author: egastal@siu.edu
Fax: +1(618) 453-5231
Received: August 1, 2013
Accepted: August 8, 2013

older mares; 4) rate of atresia increases with follicle class; 5) rate of morphologically normal follicles and follicle class proportions are similar between tissue chopper (mechanical isolation) and histological analyses; 6) rate of morphologically normal follicles, follicle viability, and follicle class proportions are similar between follicles from *in vitro* BPU fragments and scalpel blade dissected fragments; 7) a substantial number of follicles are lost during mechanical isolation using a tissue chopper compared to *in situ* histological analysis, regardless of the methodology used for harvesting tissue (i.e. *in vitro* BPU vs. scalpel blade); 8) preantral follicles submitted to an *in vitro* culture system will respond positively by undergoing activation and growth while remaining morphologically normal; and 9) preantral follicles will respond differently depending on the base medium (α-MEM or TCM-199) used for *in vitro* culture. This article will review some of the important findings of our recent studies related to the harvesting, processing, and culture of equine preantral follicles and discuss those with the limited information available in the literature.

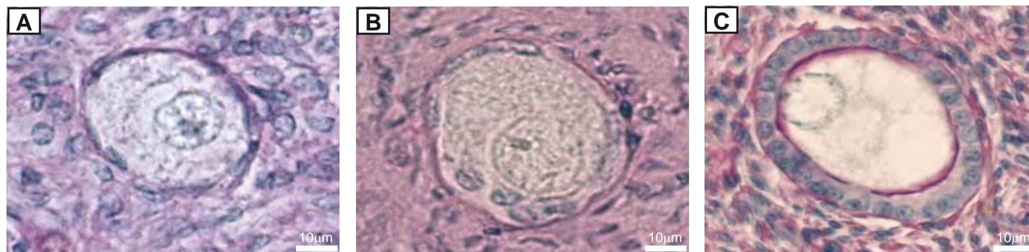


Figure 1. Morphology of equine preantral follicles. Normal (A) primordial, (B) transitional, and (C) primary follicles.

Early folliculogenesis

Folliculogenesis, or the complete development of ovarian follicles and their respective oocytes from the early primordial phase through complete maturation, is a vital feature of female fertility and reproduction in general. However, the mechanisms that control this phenomenon are not well understood, especially during the earlier stages of folliculogenesis. Recent technologies, including *in vitro* preantral follicle culture, are beginning to give insight into how early follicle activation and growth occur.

It has long been accepted that female mammals are born with a finite pool of resting follicles which constitute the ovarian reserve (Gougeon, 2010). However, recent research suggests that the mammalian ovary may be capable of generating new oocytes after birth (White *et al.*, 2012). Regardless, the follicle population decreases with age as the follicles enter into the growth phase, or the more likely result, undergo follicular atresia (Eppig and O'Brien, 1996). Primordial (one layer of flattened granulosa cells around the oocyte), transitional (both flattened and cuboidal granulosa cells around the oocyte), and primary (one layer of cuboidal granulosa cells around the oocyte) follicles make up a large majority of the total ovarian reserve (Silva *et al.*, 2004b). The ovarian follicle population differs greatly among species, but seems to follow the same trend as the population reaches its peak during fetal life, diminishes greatly by parturition, and continues to be depleted well into adult life. Rat ovaries are estimated to contain 64,000 normal follicles/oogonia at 17.5 days post-conception, 39,000 at birth, and 19,000 by 2 days post-partum (Beaumont and Mandl, 1962). A similar trend is seen in humans where the total follicle/oocyte population reaches its peak of 6,800,000 at 5 months post-conception, drops to 2,000,000 at birth, and reaches 300,000 by 7 yr post-partum (Baker, 1963; Wise *et al.*, 1996). Preantral follicle populations have not been studied in the fetal or neo-natal horse ovary, but the ovaries of 2- to 4-yr-old pony and saddle-type mares contain an average of 35,590 primordial follicles (Driancourt *et al.*, 1982). Since mares only naturally ovulate an average of 12.4 times per year (Kot and Tischner, 1991), it is obvious that a huge majority (>99%) of primordial follicles from the initial ovarian reserve will never reach the ovulatory phase.

Very little is known about what causes preantral follicles to enter the growth phase. Studies showing that early folliculogenesis is normal in women with follicle-stimulating-hormone (FSH) deficiency (Kumar *et al.*, 1997) and in both FSH-β subunit (Matthews *et al.*, 1993) and FSH-receptor knock-out (KO) mice (Dierich *et al.*, 1998) suggest that this process is gonadotropin independent. However, receptors for FSH have been identified in the granulosa cells of primary, secondary, and antral follicles in cattle (Wandji *et al.*, 1992) and hamsters (Roy and Albee, 2000), and in oocytes (Méduri *et al.*, 2002; Durlej *et al.*, 2011) of primordial and primary follicles and granulosa cells of primary follicles in swine (Durlej *et al.*, 2011). Therefore, it is possible that FSH can directly and/or indirectly stimulate primordial follicle growth by causing the release of various factors from later stage follicles or stroma cells that act in a paracrine fashion, such as IGF-I and activin (van den Hurk and Zhao, 2005). Several other molecules have been suggested to either inhibit or stimulate preantral follicle growth (for review see Monget *et al.*, 2012), but the specific details of this process remain unknown.

Summary of recent equine preantral follicle studies

For a better understanding, and to facilitate the discussion of our results with the pertinent available literature, presented below are brief summaries of the results from our studies on equine preantral follicles (Haag *et al*., 2013a, b, c).

Study 1. Quantification, morphology, and viability of equine preantral follicles obtained via the biopsy pick-up method

Due to the difficulty of obtaining equine ovarian tissue, a method for repeated collection of preantral follicles was tested in mares (Haag *et al*., 2013c). The goals of this study were to refine the transvaginal, ultrasound-guided BPU method for ovarian stroma in mares and to assess the number, viability, and morphology of preantral follicles harvested. A total of 33 ovarian biopsy procedures were performed on 18 mares during the breeding season. Mares were 5- to 21-yr-old and biopsies were performed during the estrous and/or diestrous phase as confirmed by transrectal ultrasonography. Follicles were isolated mechanically using a tissue chopper, counted and classified, measured for follicle and oocyte diameter, and analyzed for either viability or morphology. A total of 256 biopsy attempts (n = 8 biopsy attempts per biopsy procedure) were made resulting in 185 successful tissue sample collections (72% success rate). The mean weight of ovarian tissue collected per procedure was 25 mg. Overall, 620 preantral follicles were collected and isolated (95% primordial and 5% primary; Table 1). An average of 19 follicles were isolated per biopsy procedure. Primordial and primary follicles had an average diameter of 31 and 42 µm, respectively. Viability rate (as assessed using Trypan Blue dye) was higher for primordial follicles (91%) compared to primary follicles (50%). Primordial follicles tended to have a higher rate of morphological normality (96%) compared to primary follicles (80%). The total number of follicles isolated, amount of tissue harvested, and number of follicles per mg of tissue did not differ according to phase of the estrous cycle (Table 2). Younger mares (ages 5 to 7 yr) had more follicles isolated per procedure than older mares (ages 14 to 21 yr). The length of the interovulatory interval was not affected by any biopsy procedure, and no adverse effects in cyclicity or general reproductive health were observed. We concluded that the BPU method provided satisfactory numbers of normal and viable preantral follicles for the study of early follicular development in the equine species.

Study 2. Equine preantral follicles obtained via the biopsy pick-up method: histological evaluation and validation of a mechanical isolation technique

The purposes of this study (Haag *et al*., 2013a) in mares were to 1) compare preantral follicle parameters between *in vitro* BPU and scalpel blade dissection collection methods and between histological and mechanical isolation (tissue chopper) processing techniques (Experiment 1); 2) histologically evaluate preantral follicles (Experiment 2); and 3) compare histological analysis with a previously established mechanical isolation technique for ovarian cortical fragments obtained *in vivo* using a BPU instrument (Experiment 3). For Experiment 1, a total of 220 preantral follicles were analyzed (90% primordial and 10% primary). Proportions of primordial and primary follicles did not differ between tissue collection (BPU vs. scalpel blade dissection) or processing (mechanical isolation vs. histology) methods. Follicle viability and morphological normality rates were similar between tissue collection methods (Fig. 2). For Experiment 2, a total of 332 preantral follicles were analyzed. Primordial and transitional (combined) follicles and oocytes averaged 36 and 26 µm in diameter, respectively, whereas primary follicles and oocytes averaged 43 and 32 µm in diameter, respectively. For Experiment 3, a total of 188 preantral follicles were analyzed. The proportion of primordial versus primary follicles was higher for histological analysis (98%) compared to tissue chopper analysis (94%) within the same animals. Number of follicles per mg of tissue was not different within animals when the processing methods were compared. We concluded that most parameters evaluated for preantral follicles were similar between histological and tissue chopper processing techniques, indicating that mechanical isolation is an efficient way to dissociate preantral follicles from the equine ovarian cortex.

Table 1. Diameter and number of follicles and oocytes harvested per BPU procedure.

	Primordial follicles (n)	Primordial follicle diameter (µm)$^{\Psi}$	Primordial oocyte diameter (µm)$^{\Psi}$	Primary follicles (n)	Primary follicle diameter (µm)$^{\Psi}$	Primary oocyte diameter (µm)$^{\Psi}$	Total preantral follicles (n)
Mean ± SEM	17.8 ± 1.7[a]	31.0 ± 0.5[c]	27.6 ± 0.6[e]	1.0 ± 0.2[b]	42.3 ± 1.6[d]	34.4 ± 0.7[f]	18.8 ± 1.9
Total (n)	588	277	277	32	11	11	620
% of total	94.8			5.2			100

$^{\Psi}$Diameter was measured on morphologically normal follicles/oocytes only. [a,b;c,d;e,f]Same end points between primordial and primary follicles/oocytes without a common superscript differed (P < 0.05). Adapted from Haag *et al*. (2013c).

Table 2. Mean (± SEM) diameter, total number, viability, and morphology of preantral follicles harvested per BPU procedure.

	Primordial follicle diameter (µm)[†]	Primary follicle diameter (µm)[Ψ]	Total preantral follicles (n)[§,‡]	Viability (% live)[‡]	Morphology (% normal)[‡]
Age groups					
Younger (6.0 ± 0.3 yr, n=6)	30.5 ± 0.7[a]	38.2 ± 1.9	26.6 ± 4.7[a]	90.7 ± 2.6[a]	97.9 ± 1.9[a]
Older (16.0 ± 0.8 yr, n=11)	31.0 ± 1.1[a]	44.9 ± 3.9	15.5 ± 2.8[b]	82.9 ± 4.4[a]	94.0 ± 3.4[a]
Phase of estrous cycle groups					
Estrous (n=15)	30.0 ± 0.8[a]	40.6 ± 2.2	15.9 ± 2.6[a]	91.1 ± 3.7[a]	96.0 ± 1.9[a]
Diestrous (n=18)	31.8 ± 0.9[a]	41.7 ± 2.5	21.3 ± 3.4[a]	87.6 ± 2.5[a]	94.9 ± 2.5[a]

[†]Number of primordial follicles evaluated ranged from 67 to 117 (age groups) and 116 to 157 (phase of estrous cycle groups). [Ψ]Not analyzed due to the low number of observations (n = 2 to 6). [§]Includes primordial and primary follicles. The total number of preantral follicles evaluated for younger versus older mares was 239 and 170, respectively. [‡]Comparison between estrous and diestrous phases was performed using 14 mares in consecutive phases of the estrous cycle. The number of preantral follicles evaluated was 223 and 298 for estrous and diestrous phases, respectively. [a,b]Within a column, values between age groups and between phase of the estrous cycle groups without a common superscript differed ($P < 0.05$). Adapted from Haag *et al.* (2013c).

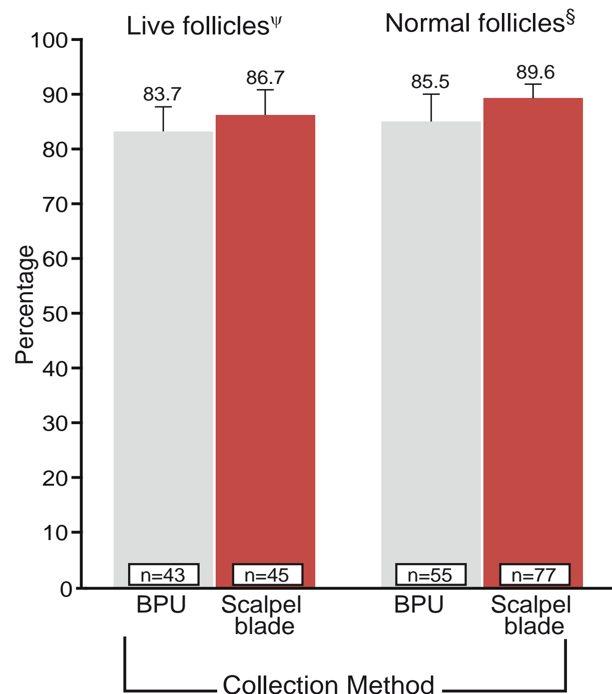


Figure 2. Overall viability and morphology rates comparing tissue collection (BPU vs. scalpel blade) methods. [Ψ]Data from tissue chopper analysis only. [§]Combined data from tissue chopper and histological analyses. Within each end point, collection methods did not differ ($P > 0.05$).

Study 3. In vitro culture of equine preantral follicles obtained via the biopsy pick-up method

The goals of this study (Haag *et al*., 2013b) were to 1) study folliculogenesis in mares by developing an *in vitro* culture system for preantral follicles obtained via the BPU method; and 2) determine which culture medium (α-MEM or TCM-199) was most efficient for promoting follicular growth and development and maintaining morphologically normal follicles throughout

1 and 7 days of culture. Ovarian cortical strips were obtained from 5- to 16-yr-old mares (n = 10) via the BPU method (n = 10 procedures) during the breeding season. The base culture media were supplemented with glutamine, hypoxanthine, bovine serum albumin, insulin, transferrin, selenium, ascorbic acid, penicillin, and streptomycin and were named α-MEM$^+$ and TCM-199$^+$. Ovarian tissue was immediately submitted to histological analysis (noncultured control; D0) or cultured *in situ* for 1 day (D1) or 7 days (D7) in either α-MEM$^+$ or TCM-199$^+$ and submitted to histological analysis, generating five treatment groups: noncultured control, α-MEM:D1, TCM-199:D1, α-MEM:D7, and TCM-199:D7. A total of 142 preantral follicles were analyzed in five replicates. No follicles were observed in the TCM-199:D7 treatment group. The proportion of primordial follicles was higher in the control group compared to the α-MEM:D7 treatment group. The proportion of primary follicles was higher in the α-MEM:D7 treatment group compared to the control. In addition, the proportion of developing follicles (transitional, primary, and secondary) was higher in the α-MEM:D7 treatment compared to the control group (Fig. 3). These results indicate that follicular activation did occur. There was no difference in the percentages of normal primordial and primary follicles among treatments. A higher percentage of normal developing follicles was observed in the α-MEM:D1 treatment compared to the TCM-199:D1 and α-MEM:D7 treatment groups. Overall, the percentage of normal follicles was higher in the control (72%) and α-MEM:D1 (84%) treatments compared to the α-MEM:D7 (27%) treatment group. Mean follicle diameter was greater in the α-MEM:D7 treatment (41 µm) compared to the control group (37 µm). Mean oocyte diameter (31-33 µm) was greater in the α-MEM:D1, TCM-199:D1, and α-MEM:D7 treatments compared to the control group (27 µm). We concluded, based on these preliminary data, that the *in vitro* culture of equine ovarian fragments obtained *in vivo* via the BPU method promoted preantral follicle development and follicle and oocyte growth in α-MEM$^+$ for 7 days, with some follicles maintaining morphological normality throughout the culture period.

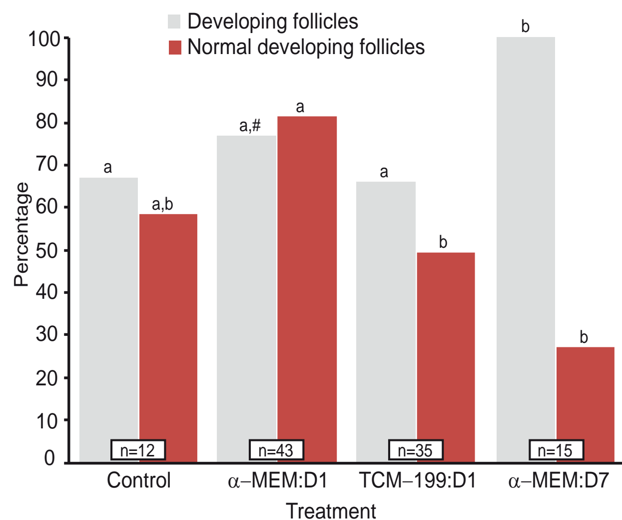


Fig. 3. Percentage and morphology of developing follicles in culture. Among treatment groups, bars for each end point without a common letter differed (P < 0.05). #Tended (P < 0.06) to differ from the α-MEM:D7 treatment group. Only developing follicles were observed in the α-MEM:D7 treatment group. No follicles were observed in the TCM-199:D7 treatment group.

Limitations to studying early folliculogenesis in the equine species

Folliculogenesis begins when resting follicles from the ovarian reserve are activated for growth and terminates when a follicle reaches the ovulatory phase. This process can be broken down into four basic steps (for review see Gougeon, 2010): 1) initiation; 2) early follicle growth; 3) follicle selection; and 4) maturation of the preovulatory follicle. Tools such as transrectal ultrasonography and follicle aspiration have allowed for extensive studies on follicle selection and maturation of the preovulatory follicle in the equine species (for reviews see Gastal, 2009, 2011). However, these technologies are limited to the study of antral follicles. This barrier highlights the importance of introducing new technologies that allow for the study of early folliculogenesis in mares.

Having access to ovarian tissue is one of the main limiting factors in studying early folliculogenesis in any species. A majority of studies in the literature use preantral follicles that were harvested from whole ovaries

acquired from individuals that were slaughtered, euthanized, or died unexpectedly, or through ovariectomy. This presents a problem in the equine species, for which abattoirs are not as abundant as for other species.

Biopsy Pick-Up method

In cases where whole ovaries are scarce or unavailable, the BPU method provides a good alternative, and even has some advantages. Whereas whole ovaries are only available from dead or ovariectomized individuals, the BPU method offers the advantage of being able to repeatedly harvest preantral follicles from live individuals, apparently without jeopardizing their short-term reproductive function or general health. This technology was originally introduced in humans as a means to evaluate the ovarian reserve of preantral follicles using ovarian cortical biopsies (Lass *et al*., 1997). The BPU method has been used to harvest preantral follicles from women (Lass *et al.*, 1997; Meirow *et al.*, 1999; Qu *et al.*, 2000; de Bruin *et al.*, 2002; Schmidt *et al.*, 2003; Rice *et al.*, 2008; Zhou *et al.*, 2010; David *et al.*, 2011) and cows (Aerts *et al.*, 2005, 2008). Studies in women have used ovarian cortical biopsies to evaluate the ovarian reserve of preantral follicles (Lass *et al*., 1997; Schmidt *et al.*, 2003), to analyze cryopreservation techniques to preserve fertility (Meirow *et al.*, 1999; Qu *et al.*, 2000; Zhou *et al.*, 2010), and to evaluate the ultrastructure of preantral follicles (de Bruin *et al.*, 2002). Preserving fertility in women whose resting follicle reserve is threatened by medical procedures such as chemotherapy and/or radiotherapy treatment is one of the ultimate goals associated with ovarian biopsy. Even though human preantral follicles from ovarian biopsy tissue have been successfully cryopreserved, indicated by no difference in follicle morphology before or after cryopreservation (Qu *et al.*, 2000), a preantral follicle culture system that can facilitate complete folliculogenesis has not been established in this species.

Aerts *et al.* were the first group to use the transvaginal BPU method to harvest preantral follicles from a live animal (cow) to evaluate the effects of BPU on post-mortem ovaries (Aerts *et al.*, 2005) and to quantify and analyze the viability of preantral follicles (Aerts *et al.*, 2008). These two bovine studies revealed that the vast majority (93%) of follicles analyzed were considered viable and suitable for culture. Additionally, a post-mortem analysis of the repeatedly biopsied ovaries revealed neither adhesions nor morphological abnormalities and no evidence of scar tissue formation. Regular ovarian activity continued after the biopsy procedure was performed as evidenced by ultrasonographic examination in live animals and the presence of several new antral follicles on post-mortem ovaries. While a post-mortem analysis of the biopsied ovaries was not possible in our recent study (Haag *et al.*, 2013c), continued ultrasound scanning of the ovaries well after the BPU procedures was performed and revealed resumed normal cyclicity in all mares, with no change in interovulatory interval before, during, or after the BPU procedure(s). In addition, no visible changes in the ovaries were apparent during later ultrasound examinations. A gross and microscopic examination of biopsied mare ovaries would be very helpful in determining any possible short- or long-term effects of the BPU procedure on the ovaries.

Due to the closing of the last equine abattoir in the United States in 2007, ovarian tissue has been difficult to obtain in recent years for studies on early folliculogenesis in mares. Therefore, it is easy to realize the importance of the BPU method to get around this problem. Not only does the procedure provide adequate numbers of preantral follicles (Haag *et al*., 2013a, b, c), but it can also be performed repeatedly on the same individuals. This could allow for future studies that analyze preantral follicle populations continuously throughout a mare's lifetime, studying the effect of age or other factors, such as pathologies, on the resting follicle reserve. The alternative approaches (ovaries obtained from dead or ovariectomized mares) for harvesting preantral follicles are not repeatable within individuals and would not allow for the same types of dynamic studies.

Not only does the BPU method provide satisfactory numbers of preantral follicles, but it also provides mostly morphologically normal and viable follicles, indicating their suitability for *in vitro* culture. The overall follicle viability rates observed in our recent studies (85.2 to 91.1%; Haag *et al*., 2013a, b, c) are similar to those seen in cows for preantral follicles obtained via the BPU method (92.8%; Aerts *et al*., 2008). A comparison between preantral follicles from *in vitro* BPU fragments and scalpel blade dissected fragments showed no difference in follicle morphology or viability according to collection method (Haag *et al*., 2013a), suggesting that the BPU procedure itself does not affect those parameters.

Preantral follicle isolation

In order to analyze preantral follicles (except for *in situ* histological analysis), they must first be isolated from the surrounding ovarian tissue. Preantral follicles have been released from the ovarian stroma of several species using either enzymatic (human: Roy and Treacy, 1993; bovine: Carámbula *et al*., 1999; murine: Demeester *et al*., 2002) or mechanical isolation (bovine: Nuttinck *et al*., 1993; caprine: Lucci *et al*., 1999a, b; ovine: Amorim *et al*., 2000), or a combination of both methods (bovine: Figueiredo *et al*., 1993). Our recent studies (Haag *et al*., 2013a, c) are the first in the equine species to dissociate preantral follicles from the ovarian

stroma using a mechanical isolation technique.

In our recent studies (Haag *et al*., 2013a, c), the mechanical approach was preferred over enzymatic isolation because of results from previous enzymatic isolation studies in this species. Apparently, only three studies exist in which equine preantral follicles were isolated from the ovarian stroma, and all three studies employed enzymatic isolation using collagenase (Telfer and Watson, 2000) or collagenase in combination with DNase (Szlachta and Tischner, 2000, 2004). Telfer and Watson (2000) reported that 70% of all follicles had diameters between 90 and 150 µm after incubation with collagenase, and Szlachta and Tischner (2000, 2004) reported follicle diameters between 60 and 220 µm after incubation with collagenase and DNase. These diameters indicate that most follicles isolated enzymatically were most likely beyond the primordial or primary stage of development, which averaged 31 and 42 µm in diameter, respectively, in our recent studies. In addition, Telfer and Watson (2000) were able to isolate only a small number of preantral follicles (~30 to 60) per ovary. Since primordial follicles are considered to be the prime starting material for *in vitro* culture (Cortvrindt and Smitz, 2001), mechanical isolation was used in our studies (Haag *et al*., 2013a, c) based on previous studies in other species that isolated high proportions of primordial follicles using a tissue chopper (bovine: Nuttinck *et al*., 1993; caprine: Lucci *et al*., 1999a, b; ovine: Amorim *et al*., 2000). The tissue chopper has been utilized by several research groups around the world and is considered a practical and fast way to mechanically isolate large numbers of viable and morphologically normal preantral follicles.

In our recent studies (Haag *et al*., 2013a, c), not only did mechanical isolation provide a large number of preantral follicles with a high proportion of primordial follicles, but also the vast majority of the follicles were morphologically normal and viable. A previous study which analyzed extracellular matrix proteins in bovine preantral follicles showed the presence of collagen fibers in the basement membrane of preantral follicles and led the authors to believe that a mechanical defense was provided by the collagen fibers, protecting the follicle from physical damage during the mechanical isolation process (Figueiredo *et al*., 1995). This is extremely important as the success of future technologies involving the further use of preantral follicles depends on the ability to begin with healthy follicles. It should be noted, however, that mechanical isolation using a tissue chopper has been associated with a high percentage of preantral follicles lost during the process. In studies analyzing recovery rates for preantral follicles mechanically isolated from whole ovary slices by comparing the data with *in situ* histological analysis, overall recovery rates were 28, 35, and 46% (36% overall) for prepubertal, nonpregnant adult, and pregnant adult caprine ovaries, respectively (Lucci *et al*., 1999b), and 5, 28, and 26% (17% overall) for fetal, nonpregnant adult, and pregnant adult ovine ovaries, respectively (Amorim *et al*., 2000). Although in our study (Haag *et al*., 2013a) there was no statistical difference in the number of follicles per mg of tissue comparing tissue chopper and histological analyses within animals, the number of follicles per mg of tissue was 34% lower for tissue chopper analysis (recovery rate: 66%). A study using larger numbers of equine preantral follicles from whole ovary slices (not just BPU fragments) might reveal a statistical difference between the two processing methods.

Even though enzymatic isolation using collagenase showed no deleterious effect on follicle morphology in mares (Telfer and Watson, 2000), that study and the other two studies in mares (Szlachta and Tischner, 2000, 2004) did not evaluate follicle viability or oocyte quality, and oocyte quality has been shown to be negatively affected by collagenase treatment in cows (Wandji *et al*., 1996). However, Aerts *et al*. (2008) used collagenase to enzymatically isolate bovine preantral follicles from ovarian fragments obtained via BPU and observed an overall follicle viability rate of 92.8%. Further studies are necessary to compare the effects of enzymatic versus mechanical isolation of preantral follicles from equine ovarian tissue.

Histological analysis

Only a few studies have evaluated equine preantral follicles using histological analysis for the purposes of estimating ovarian follicle populations (Driancourt *et al*., 1982), evaluating preantral follicle morphology after enzymatic isolation (Telfer and Watson, 2000), and studying the distribution, morphology, and ultrastructure of preantral follicles (Szlachta and Tischner, 2002). All these studies used hematoxylin and eosin for staining and used sectioning intervals of 10, 7, and 2 µm, respectively.

In our recent studies (Haag *et al*., 2013a, b), tissue was cut at a 10 µm sectioning interval and was stained with Periodic-acid Schiff (PAS) and counterstained with hematoxylin. PAS was chosen for its superior ability to stain glycoproteins, which is the main component of the zona pellucida surrounding the oocyte. A 10 µm cut was chosen mainly as a matter of practicality, as a smaller cut would have resulted in many more serial sections to be analyzed. Depending on the goals of future studies, a thinner cut might be recommended, but for our purposes the 10 µm cut was adequate.

In vitro culture of preantral follicles

One of the best tools available to give insight into early folliculogenesis is *in vitro* culture of preantral follicles. Although *in vitro* culture of preantral follicles

has been moderately successful in some species, attempts with large domestic farm animals have, for the most part, been less productive. The most successful culture systems have been for mice, where primordial follicles cultured *in vitro* then subsequently fertilized have produced embryos that were transferred into recipients that gave live birth (Eppig and O'Brien, 1996; O'Brien *et al.*, 2003; Wang *et al.*, 2011). Cultured preantral follicles have resulted in the production of viable embryos in several other species, including rats (Daniel *et al.*, 1989), pigs (Wu *et al.*, 2001), buffalo (Gupta *et al.*, 2008), sheep (Arunakumari *et al.*, 2010), and goats (Magalhães *et al.*, 2011).

Systems for oocyte culture have been established in mares (for review see Hinrichs, 2010), but only two studies exist on *in vitro* culture of equine preantral follicles. In an abstract by Szlachta and Tischner (2000), enzymatically isolated follicles cultured *in vitro* responded to FSH supplementation by experiencing an increase in growth rate after 1 day of culture followed by an increased rate of atresia thereafter until day 4 of culture. Szlachta and Tischner (2004) tested the efficacy of two different culture media (Menezo B2 and Wymouth MB 752/1) with and without the supplementation of FSH on the *in vitro* culture of preantral follicles isolated enzymatically. The results showed that Menezo B2 was the superior culture medium, while supplementation with FSH neither affected follicle growth rate nor increased follicle survival rates during the 4 day culture period. While these studies do offer some insight into determining the framework of a successful *in vitro* culture system for preantral follicles in the equine species, several additional *in vitro* culture studies are necessary to establish a good system for studying early folliculogenesis in mares.

Our recent work (Haag *et al*., 2013b) is only the third known report on *in vitro* culture and the first known report on *in situ* culture of equine preantral follicles. The data generated from this preliminary study indicated that culturing preantral follicles *in situ* for 7 days in α-MEM$^+$ promoted follicle development and follicle and oocyte growth, with 27% of the follicles maintaining morphological normality throughout the culture period. Several advantages related to *in situ* culture have been outlined, as reviewed by Picton *et al*. (2008) as follows: 1) damage caused to the follicle during isolation is avoided, 2) the chances of necrosis are minimized as the surface area of the tissue is maximized for gaseous exchange and fulfillment of nutrient requirements, and 3) a complex support system is provided that closely resembles the ovarian environment *in vivo*, keeping the follicles intact and in contact with the surrounding stromal cells which trigger the initiation of follicle growth under the control of local biochemical pathways. The BPU method is a feasible way to harvest ovarian fragments with a high proportion of primordial follicles that can be directly submitted to culture without any additional manipulation or preparation, making it less time consuming than individually isolating follicles before culture, which could take several hours (Figueiredo *et al*., 2011). These advantages have made *in situ* culture a successful means of primordial follicle activation and growth in several species, including mice (Eppig and O'Brien, 1996; O'Brien *et al*., 2003), hamsters (Yu and Roy, 1999), baboons (Fortune *et al*., 1998), humans (Hovatta *et al*., 1999; Wright *et al*., 1999; Telfer *et al*., 2008), goats (Silva *et al*., 2004a, b; Martins *et al*., 2008), and cows (Wandji *et al*., 1996; Fortune *et al*., 1998; Gigli *et al*., 2006; McLaughlin and Telfer, 2010). However, the surrounding cortical tissue might act as a barrier to medium perfusion during *in situ* culture, resulting in the development of primordial follicles only to the secondary stage (Martins *et al*., 2008). Once the secondary stage is reached, those follicles can be isolated mechanically and further cultured individually to the antral stage (Eppig and O'Brien 1996; O'Brien *et al*., 2003; McLaughlin and Telfer, 2010).

The superiority of α-MEM$^+$ as a base culture medium to TCM-199$^+$ was indicated in our study (Haag *et al*., 2013b) as α-MEM$^+$ had a higher proportion of morphologically normal follicles after 1 day of culture and was the only medium in which follicles were found after 7 days of culture. The rich formulation of α-MEM has been acknowledged and it has been used as a base medium for successful *in vitro* culture systems for preantral follicles in several species, including humans (Wright *et al*., 1999), mice (Mousset-Simeón *et al*., 2005), buffalo (Gupta *et al*., 2008), dogs (Serafim *et al*., 2010), and goats (Magalhães *et al*., 2011). The additional supplementation of glutamine, hypoxanthine, BSA, ITS, and ascorbic acid has been shown to be essential for the survival of caprine preantral follicles during *in vitro* culture (Silva *et al*., 2004a). In future studies, the effects of various hormones and growth factors need to be tested on equine preantral follicles in order to determine the optimal environment for mediating complete folliculogenesis *in vitro*.

Conclusions

Our recent studies on equine preantral follicles (Haag *et al*., 2013a, b, c) produced the following general findings: 1) the BPU method provided sufficient material for the study of early folliculogenesis in mares; 2) preantral follicle quantity, morphology, and viability did not differ according to phase of the estrous cycle; 3) number of follicles, but not follicle morphology or viability, was greater for younger versus older mares; 4) rate of atresia generally increased with follicle class; 5) proportion of primordial to primary follicles was higher for histological versus tissue chopper analysis, even though overall follicle morphology rates were similar;

6) rate of morphologically normal follicles, follicle viability, and follicle class proportions were similar between follicles from *in vitro* BPU and scalpel blade dissected fragments; 7) number of follicles per mg of tissue within animals was similar between tissue chopper and histological analyses; 8) preantral follicles submitted to an *in vitro* culture system appeared to respond positively by undergoing activation and growth during a 7 day culture period, with some follicles remaining morphologically normal; and 9) α-MEM$^+$ seemed to be superior to TCM-199$^+$ as a base culture medium for equine preantral follicles.

In conclusion, the results from our recent studies validated the transvaginal, ultrasound-guided BPU method as a way to harvest ovarian fragments containing large numbers of morphologically normal and viable preantral follicles for the study of early folliculogenesis in the equine species. The ovarian fragments can be submitted to histology or processed using a tissue chopper for further analysis of the preantral follicles. The fragments can also be submitted directly to *in vitro* culture, where follicle growth and development can be achieved after 7 days of culture in α-MEM$^+$ medium. Finally, the BPU method can be used to repeatedly harvest large numbers of primordial and primary follicles from equine ovaries without jeopardizing short-term reproductive function. Successful *in vitro* culture and cryopreservation systems for this species might make the BPU method a feasible technique to provide material to enable the utilization of oocytes within the abundant preantral follicles present in the equine ovary, many of which are essentially wasted *in vivo* through inactivation or atresia during development. In the future, these technologies could potentially provide a means for the preservation of genetic material from valuable individuals or for large-scale embryo production.

References

Aerts JM, Oste M, Bols PE. 2005. Development and practical applications of a method for repeated transvaginal, ultrasound guided biopsy collection of the bovine ovary. *Theriogenology*, 64:947-957.

Aerts JM, Martinez-Madrid B, Flothmann K, De Clercq JB, Van Aelst S, Bols PE. 2008. Quantification and viability assessment of isolated bovine primordial and primary ovarian follicles retrieved through a standardized biopsy pick-up procedure. *Reprod Domest Anim*, 43:360-366.

Amorim CA, Lucci CM, Rodrigues APR, Carvalho FCA, Figueiredo JR, Rondina D, Cecchi R, Giorgetti A, Martini A, Gonçalves PBD. 2000. Quantitative and qualitative analysis of the effectiveness of a mechanical method for the isolation of preantral follicles from ovine ovaries. *Theriogenology*, 53:1251-1262.

Arunakumari G, Shanmugasundaram N, Rao VH. 2010. Development of morulae from the oocytes of cultured sheep preantral follicles. *Theriogenology*, 74:884-894.

Baerwald AR. 2009. Human antral folliculogenesis: what we have learned from the bovine and equine models. *Anim Reprod*, 6:20-29.

Baker TG. 1963. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond B Biol Sci*, 158:417-433.

Beaumont HM, Mandl AM. 1962. A quantitative and cytological study of oogonia and oocytes in the foetal and neonatal Rat. *Proc R Soc Lond B Biol Sci*, 155:557-559.

Carámbula SF, Gonçalves PB, Costa LF, Figueiredo JR, Wheeler MB, Neves JP, Mondadori RG. 1999. Effect of fetal age and method of recovery on isolation of preantral follicles from bovine ovaries. *Theriogenology*, 52:563-571.

Cortvrindt R, Smitz J. 2001. In vitro follicle growth: achievements in mammalian species. *Reprod Domest Anim*, 36:3-9.

Daniel SAJ, Armstrong DT, Gorelangton RE. 1989. Growth and development of rat oocytes in vitro. *Gam Res*, 24:109-121.

David A, Dolmans MM, Van Langendonckt A, Donnez J, Amorim CA. 2011. Immunohistochemical localization of growth factors after cryopreservation and 3 weeks' xenotransplantation of human ovarian tissue. *Fertil Steril*, 95:124-126.

de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M, Looman CW, te Velde ER. 2002. Ultrastructure of the resting ovarian follicle pool in healthy young women. *Biol Reprod*, 66:1151-1160.

Demeestere I, Delbaere A, Gervy C, Van Den Bergh M, Devreker F, Englert Y. 2002. Effect of preantral follicle isolation technique on in-vitro follicular growth, oocyte maturation and embryo development in mice. *Hum Reprod*, 17:2152-2159.

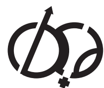
Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P. 1998. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc Natl Acad Sci USA*, 95:13612-13617.

Driancourt MA, Paris A, Roux C, Mariana JC, Palmer E. 1982. Ovarian follicular populations in pony and saddle-type mares. *Reprod Nutr Dev*, 22:1035-1047.

Durlej M, Knapczyk-Stwora K, Duda M, Galas J, Slomczynska M. 2011. The expression of FSH receptor (FSHR) in the neonatal porcine ovary and its regulation by flutamide. *Reprod Domest Anim*, 46:377-384.

Eppig JJ, O'Brien MJ. 1996. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod*, 54:197-207.

Figueiredo JR, Hulshof SCJ, Van den Hurk R, Ectors FJ, Fontes RS, Nusgens B, Bevers MM,



Beckers JF. 1993. Development of a combined new mechanical and enzymatic method for the isolation of intact preantral follicles from fetal, calf and adult bovine ovaries. *Theriogenology*, 40:789-799.

Figueiredo JR, Hulshof SCJ, Thiry M, Van den Hurk R, Bevers MM, Nusgens B, Beckers JF. 1995. Extracellular matrix proteins and basement membrane: their identification in bovine ovaries and significance for the attachment of cultured preantral follicles. *Theriogenology*, 43:845-858.

Figueiredo JR, Rodrigues AP, Silva JR, Santos RR. 2011. Cryopreservation and in vitro culture of caprine preantral follicles. *Reprod Fertil Dev*, 23:40-47.

Fortune JE, Kito S, Wandji SA, Srsen V. 1998. Activation of bovine and baboon primordial follicles in vitro. *Theriogenology*, 49:441-449.

Gastal EL. 2009. Recent advances and new concepts on follicle and endocrine dynamics during the equine periovulatory period. *Anim Reprod*, 6:144-158.

Gastal EL. 2011. Ovulation: Part 2. Ultrasonographic morphology of the preovulatory follicle. *In*: McKinnon A, Squires E, Vaala W, Varner D. (Ed.). *Equine Reproduction.* 2nd ed. Ames, IA: Blackwell. pp. 2032-2054.

Gigli I, Byrd DD, Fortune JE. 2006. Effects of oxygen tension and supplements to the culture medium on activation and development of bovine follicles in vitro. *Theriogenology*, 66:344-353.

Ginther OJ, Gastal EL, Gastal MO, Bergfelt DR, Baerwald AR, Pierson RA. 2004. Comparative study of the dynamics of follicular waves in mares and women. *Biol Reprod*, 71:1195-1201.

Ginther OJ, Beg MA, Gastal EL, Gastal MO, Baerwald AR, Pierson RA. 2005. Systemic concentrations of hormones during the development of follicular waves in mares and women: a comparative study. *Reproduction*, 130:379-388.

Ginther OJ. 2012. The mare: a 1000-pound guinea pig for study of the ovulatory follicular wave in women. *Theriogenology*, 77:818-828.

Gougeon A. 2010. Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. *Ann Endocrinol*, 71:132-143.

Gupta PSP, Ramesh HS, Manjunatha BM. 2008. Production of buffalo embryos using oocytes from in vitro grown preantral follicles. *Zygote*, 16:57-63.

Haag KT, Magalhães-Padilha DM, Fonseca GR, Wischral A, Gastal MO, King SS, Jones KL, Figueiredo JR, Gastal EL. 2013a. Equine preantral follicles obtained via the Biopsy Pick-Up method: histological evaluation and validation of a mechanical isolation technique. *Theriogenology*, 79:735-743.

Haag KT, Magalhães-Padilha DM, Fonseca GR, Wischral A, Gastal MO, King SS, Jones KL, Figueiredo JR, Gastal EL. 2013b. In vitro culture of equine preantral follicles obtained via the Biopsy Pick-Up method. *Theriogenology*, 79:911-917.

Haag KT, Magalhães-Padilha DM, Fonseca GR, Wischral A, Gastal MO, King SS, Jones KL, Figueiredo JR, Gastal EL. 2013c. Quantification, morphology, and viability of equine preantral follicles obtained via the Biopsy Pick-Up method. *Theriogenology*, 79:599-609.

Hinrichs K. 2010. The equine oocyte: factors affecting meiotic and developmental competence. *Mol Reprod Dev*, 77:651-661.

Hovatta O, Wright C, Krausz T, Hardy K, Winston RM. 1999. Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Hum Reprod*, 14:2519-2524.

Kot K, Tischner M. 1991. Embryo recovery from mares exposed to a year-to-year artificially prolonged daylength. *Theriogenology*, 36:357-365.

Kumar TR, Wang Y, Lu N, Matzuk MM. 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not for male fertility. *Nat Genet*, 15:201-204.

Lass A, Silye R, Abrams DC, Krausz T, Hovatta O, Margara R, Winston RM. 1997. Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. *Hum Reprod*, 12:1028-1031.

Lucci CM, Amorim CA, Báo SN, Figueiredo JR, Rodrigues AP, Silva JR, Gonçalves PBD. 1999a. Effect of the interval of serial sections of ovarian tissue in the tissue chopper on the number of isolated caprine preantral follicles. *Anim Reprod Sci*, 56:39-49.

Lucci CM, Amorim CA, Rodrigues APR, Figueiredo JR, Báo SN, Silva JRV, Gonçalves PBD. 1999b. Study of preantral follicle population in situ and after mechanical isolation from caprine ovaries at different reproductive stages. Anim Reprod Sci, 56:223-236.

Magalhães DM, Duarte ABG, Araújo VR, Brito IR, Soares TG, Lima IM, Lopes CA, Campello CC, Rodrigues AP, Figueiredo JR. 2011. In vitro production of a caprine embryo from a preantral follicle cultured in media supplemented with growth hormone. *Theriogenology*, 75:182-188.

Martins FS, Celestino JJ, Saraiva MV, Matos MH, Bruno JB, Rocha-Junior CM, Lima-Verde IB, Lucci CM, Báo SN, Figueiredo JR. 2008. Growth and differentiation factor-9 stimulates activation of goat primordial follicles in vitro and their progression to secondary follicles. *Reprod Fertil Dev*, 20:916-924.

Matthews CH, Borgato S, Beck-Peccoz P, Adams M, Tone Y, Gambino G, Casagrande S, Tedeschini G, Benedetti A, Chatterjee VK. 1993. Primary amenorrhoea and infertility due to a mutation in the β-subunit of follicle-stimulating hormone. *Nat Genet*, 5:83-86.

McLaughlin M, Telfer EE. 2010. Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. *Reproduction*, 139:971-978.

Méduri G, Charnaux N, Driancourt MA, Combettes

L, Granet P, Vannier B, Loosfelt, H Migrom E. 2002. Follicle-stimulating hormone receptors in oocytes? *J Clin Endocrinol Metab*, 87:2266-2276.
Meirow D, Fasouliotis SJ, Nugent D, Schenker JG, Gosden RG, Rutherford AJ. 1999. A laparoscopic technique for obtaining ovarian cortical biopsy specimens for fertility conservation in patients with cancer. *Fertil Steril*, 71:948-951.
Monget P, Bobe J, Gougeon A, Fabre S, Monniaux D, Dalbies-Tran R. 2012. The ovarian reserve in mammals: a functional and evolutionary perspective. *Mol Cell Endocrinol*, 356:2-12.
Mousset-Simeón N, Jouannet P, Le Cointre L, Coussieu C, Poirot C. 2005. Comparison of three in vitro culture systems for maturation of early preantral mouse ovarian follicles. *Zygote*, 13:167-175.
Nuttinck F, Mermillod P, Massip A, Dessy F. 1993. Characterization of in vitro growth of bovine preantral ovarian follicles: a preliminary study. *Theriogenology*, 39:811-821.
O'Brien MJ, Pendola JK, Eppig JJ. 2003. A revised protocol for in vitro development of mouse oocyte from primordial follicles dramatically improves their development competence. *Biol Reprod*, 68:1682-1686.
Picton HM, Kim S, Gosden RG. 2000. Cryopreservation of gonadal tissue and cells. *Br Med Bull*, 56:603-615.
Picton HM, Harris SE, Muruvi W, Chambers EL. 2008. The in vitro growth and maturation of follicles. *Reproduction*, 136:703-715.
Qu J, Nisolle M, Donnez J. 2000. Expression of transforming growth factor-alpha, epidermal growth factor, and epidermal growth factor receptor in follicles of human ovarian tissue before and after cryopreservation. *Fertil Steril*, 74:113-121.
Rice S, Ojha K, Mason H. 2008. Human ovarian biopsies as a viable source of pre-antral follicles. *Hum Reprod*, 23:600-605.
Roy SK, Treacy BJ. 1993. Isolation and long-term culture of human preantral follicles. *Fertil Steril*, 59:783-790.
Roy SK, Albee L. 2000. Requirement for follicle-stimulating hormone action in the formation of primordial follicles during perinatal ovarian development in the hamster. *Endocrinology*, 141:4449-4456.
Schmidt KL, Byskov AG, Nyboe Andersen A, Müller J, Yding Andersen C. 2003. Density and distribution of primordial follicles in single pieces of cortex from 21 patients and in individual pieces of cortex from three entire human ovaries. *Hum Reprod*,18:1158-1164.
Serafim MK, Araújo VR, Silva GM, Duarte AB, Almeida AP, Chaves RN, Campello CC, Lopes CA, de Figueiredo JR, da Silva LD. 2010. Canine preantral follicles cultured with various concentrations of follicle-stimulating hormone (FSH). *Theriogenology*, 74:749-755.
Silva JR, van den Hurk R, Costa SH, Andrade ER, Nunes AP, Ferreira FV, Lôbo RN, Figueiredo JR. 2004a. Survival and growth of goat primordial follicles after in vitro culture of ovarian cortical slices in media containing coconut water. *Anim Reprod Sci*, 81:273-286.
Silva JR, van den Hurk R, Matos MHT, Santos RR, Pessoa C, Moraes MO, Figueiredo JR. 2004b. Influences of FSH and EGF on primordial follicles during in vitro culture of caprine ovarian cortical tissue. *Theriogenology*, 61:1691-1704.
Szlachta M, Tischner M. 2000. Isolation and short-term culture of preantral follicles from mare ovaries. In: Proceedings of the 14th International Congress on Animal Reproduction, 2000, Stockholm, Sweden. Stockholm: ICAR, v.2, pp. 224. (abstract).
Szlachta M, Tischner M. 2002. Distribution, morphology and ultrastructure of preantral follicles in the ovary of the mare. Havemeyer Foundation Monograph Series, 5:33-35.
Szlachta M, Tischner M. 2004. Isolation and in-vitro culture of preantral follicles in mares. *Medycyna Weterynaryjna*, 60:1177-1180.
Telfer EE, Watson ED. 2000. Method for isolating preantral follicles from mare ovaries. J *Reprod Fertil Suppl*, 56:447-453.
Telfer EE, McLaughlin M, Ding C, Thong KJ. 2008. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod*, 23:1151-1158.
van den Hurk R, Zhao J. 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology*, 63:1717-1751.
Wandji SA, Pelletier G, Sirard MA. 1992. Ontogeny and cellular localization of 125I-labeled insulin-like growth factor-I, 125I-labeled follicle-stimulating hormone, and 125I-labeled human chorionic gonadotropin binding sites in ovaries from bovine fetuses and neonatal calves. *Biol Reprod*, 47:814-822.
Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. 1996. Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod*, 55:942-948.
Wang X, Catt S, Pangestu M, Temple-Smith P. 2011. Successful in vitro culture of pre-antral follicles derived from vitrified murine ovarian tissue: oocyte maturation, fertilization, and live births. *Reproduction*, 141:183-191.
White YA, Woods DC, Takai Y, Ishihara O, Seki H, Tilly JL. 2012. Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. *Nat Med*, 18:413-421.
Wise PM, Krajnak KM, Kashon ML. 1996. Menopause: the aging of multiple pacemakers. *Science*, 273:67-70.
Wright CS, Hovatta O, Margara R, Trew G, Winston RM, Franks S, Hardy K. 1999. Effects of follicle-stimulating hormone and serum substitution on

the in-vitro growth of human ovarian follicles. *Hum Reprod*, 14:1555-1562.

Wu J, Carrel DT, Wilcox AL. 2001. Development of in vitro-matured oocytes from porcine preantral follicles following intracytoplasmic sperm injection. *Biol Reprod*, 65:1579-1585.

Yu N, Roy SK. 1999. Development of primordial and prenatal follicles from undifferentiated somatic cells and oocytes in the hamster prenatal ovary in vitro: effect of insulin. *Biol Reprod*, 61:1558-1567.

Zhou XH, Wu YJ, Shi J, Xia YX, Zheng SS. 2010. Cryopreservation of human ovarian tissue: comparison of novel direct cover vitrification and conventional vitrification. *Cryobiology*, 60:101-105.