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The mare model for follicular maturation and reproductive aging in the woman

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Abstract

Reproductive aging and assisted reproduction are becoming progressively more relevant in human medicine. Research with human subjects is limited in many aspects, and consequently animal models may have considerable utility. Such models have provided insight into follicular function, oocyte maturation, and reproductive aging. However, models are often selected based on factors other than physiological or functional similarities. Although the mare has received limited attention as a model for reproduction in women, comparisons between these species indicate that the mare has many attributes of a good model. As the mare ages, cyclic and hormonal changes parallel those of older women. The initial sign of reproductive aging in both species is a shortening of the reproductive cycle with elevated concentrations of FSH. Subsequently, cycles become longer with intermittent ovulations and elevated concentrations of FSH and LH. Reproduction ceases with failure of follicular growth and elevated gonadotropins, apparently because of ovarian failure. In the older woman and mare, oocytes have been maintained in meiotic arrest for decades – approximately four to five for the woman and two to three for the mare; in both species, reduced oocyte quality is the end factor identified in age-associated infertility. After induction of oocyte maturation in vivo, the timeline to ovulation is the same for the mare and woman, suggesting a comparable sequence of events. The mare's anatomy, long follicular phase and single dominant follicle provide a foundation for studies in oocyte and follicular development. The aim of this review is to evaluate the mare as an animal model to study age-associated changes in reproduction and to improve our understanding of oocyte and follicular maturation in vivo.

Keywords: Mare; Woman; Oocyte; Reproduction; Aging; Cycle

1. Introduction

The study of follicular function and reproductive aging in women is hindered by the availability of appropriate models. Consequently, different animal models have been used, ranging from the mouse to the monkey [1–3]. Various animal models have advantages and disadvantages for studying specific areas of reproduction, particularly in regard to human reproduction. The nonhuman primate may represent one of the most appropriate models for the woman because of evolutionary relatedness and similarities in reproductive aging [4]. However, studies in nonhuman primates can be limited by availability of animals, costs, management considerations, and ethical concerns.

The majority of research on reproductive aging has incorporated rodent models, which have advantages including ease of management, space considerations, population uniformity, rapid aging, and ease of gene manipulations. Undoubtedly, rodent models are power-

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ful tools for many research applications. However, in comparison to women, rodents are multiparous, with a short life span, rapid progression of reproductive processes and differences in reproductive endocrine patterns. The uniformity in lines of rodents allows collection of samples with minimal variation between animals; however, the potential to make repeated observations or study individual variation is limited.

Aging has an unequivocal effect on human reproduction. This has become more apparent as socioeconomic changes result in women waiting until a later age to start a family. The potential for studies in the woman are limited; therefore, animal models have been used to study reproductive aging, with most such mammalian research done on rapidly aging animals, especially rodents [1–3]. Other domestic species, such as the bovine [5], have been proposed as a model, but relatively few of these animals are maintained until old age.

In contrast to many domestic species, mares are often kept until an old age ($\geq$20 years) as working or companion animals, providing a population of mares in which oocytes have been maintained in meiotic arrest for decades. Frequently, horses are housed in close proximity to humans, with exposure to many of the same environmental factors that may affect reproduction and fertility. The mare's anatomy and disposition are uniquely suited for reproductive studies. The mare's reproductive tract is easily assessable for palpation and ultrasound, with the potential for repeated observations or follicular manipulations. As in women, the mare is monovular with a long follicular phase, allowing studies of follicular growth, deviation, and regression. The aim of this paper is to evaluate the mare as a potential model to study follicular and oocyte maturation and reproductive aging in the woman.

2. Ovarian follicular activity and effects of aging

The mare is seasonally polyestrus, with regular reproductive cycles occurring during periods of long daylight and transitional intervals into and out of the ovulatory season. Although seasonality does not occur in women, this characteristic in an animal model adds the potential to examine reproductive factors when cyclic hormonal patterns are not occurring.

Younger mares and women had regular reproductive cycles, with interovulatory intervals of 21 days for mares 3 to 13 years, and 27 days for women 19 to 43 years [6]. During an interovulatory interval, major and minor follicular waves occurred, with selection of a single ovulatory follicle in most mares [7] and women [8]. Ovarian follicular waves have been compared between mares and women [6]. A number of similarities were noted, including percentage growth of follicles during the common growth phase and a 2:1 ratio for mare to woman in the diameter of the dominant follicle from deviation to ovulation. Follicular emergence and deviation was temporally associated with changing FSH concentrations in both species [9]. The rate of growth of the dominant follicle was 3 and 1 mm/day for mares and women, respectively, with preovulatory follicles growing to 45 and 22 mm, respectively [6]. These similarities in ovarian follicular activity support the use of the mare as a model to study follicular growth and regression, with the large follicles and long follicular phase in the mare providing opportunities to investigate and manipulate the events associated with follicular growth, selection, and maturation.

With aging, cyclic changes occurred that probably reflected alterations in ovarian function. The first sign of reproductive aging in women was a shortening of the menstrual cycle [10–14]. Shorter cycles were attributed to a shortening of the follicular phase in older versus younger women, with similar luteal phases and progesterone concentrations [13]. Concentrations of FSH were elevated in older women during the early follicular phase and declined as concentrations of estradiol increased, although concentrations of LH were not altered by age [13]. Soules et al. reported similar findings when cycle characteristics of women, 40–45 years with regular ovulatory cycles, were compared to women 20–25 years old [15]. The older women had shorter menstrual cycles because of shortened follicular phases. The advanced follicular development in the older woman was attributed to an earlier recruitment and selection of the dominant follicle, because of a significantly earlier rise in FSH concentrations during the late luteal phase of the preceding cycle. Although not significantly different, the mean diameter of the preovulatory follicle was 19.8 mm in the older woman and 21.9 in the younger women [15].

As in women, characteristics of reproductive cycles also changed with aging in mares. Cycle characteristics were compared between middle-aged mares (15–19 years) and young mares (5–7 years) [16]. Similar to women [13], luteal phases did not differ with mare age [16]; however, a 15% reduction in the length of the follicular phase was noted for middle-aged mares (8.0 days versus 9.4 days in middle-age and young, respectively), and they ovulated significantly smaller follicles. The age-associated changes in cycles appeared to be propagated by changing concentrations of FSH, which rose significantly earlier in the luteal phase and to higher levels for the middle-aged mares. Consequently,

these mares had more follicles of medium sizes and a larger dominant follicle at the end of the luteal phase. During the follicular phase, the follicular growth rate was significantly slower for middle-aged than young mares [16].

Elevated concentrations of FSH, without concomitant changes in LH concentrations, appeared to be the initial sign of reproductive aging in women and mares. Cyclic changes in middle-aged mares and women demonstrated similar characteristics and were suggestive of a similar etiology. Hormonal changes were hypothesized to reflect altered ovarian secretions of FSH-modulating hormones, including estradiol, inhibin, activin and follistatin [15]. The selective increase in FSH concentrations could stimulate a greater proportion of primordial follicles to enter the growing pool of follicles and cause accelerated depletion of the primordial follicle reserve [17].

The horse could provide a unique perspective in regard to the capacity of ovaries from mares of different ages to respond to FSH stimuli. Reproductive cycles in mares occurred during periods of long daylight, and most mares had inactive ovaries during winter months. During the spring transition, increased secretions of GnRH resulted in elevated concentrations of FSH, while concentrations of LH remained minimal until just prior to the first ovulation of the year; late in the transitional period, the follicles became large and competent to produce FSH inhibitory factors (review [18]). The number of follicles $\geq$10 mm in the ovaries of mares 3 to 7, 17 to 19 and $\geq$20 years were quantified for more than 2 months before the first ovulation of the year [19]. The mean number of follicles was significantly different among ages, with follicle numbers decreasing with increasing mare age. These results demonstrated an age-associated decrease in the number of follicles developing to the antral stage during a time when concentrations of FSH should have been elevated, and minimal ovarian feedback was present.

With further reproductive aging in the woman and mare, cycle length increased, and concentrations of gonadotropins rose. In women older than 43 years, the average cycle was longer and more variable in length, with elevated LH and FSH concentrations [20]. Mares $\geq$20 years had significantly longer interovulatory intervals, with similar luteal phases but longer follicular phases than young mares [16]. This was associated with an approximately 3 days delay in emergence of the dominant follicle [21] and a slower growth rate of the ovulatory follicle [16].

After elongated follicular phases, intermittent follicular growth was observed before cessation of ovarian activity. In women and mares, researchers reported periods of minimal ovarian activity during which gonadotropin concentrations remained elevated; with growth of a follicle, gonadotropin concentrations declined [22,23]. Lengths of reproductive cycles for women between 2 and 5 years prior to menopause ranged from 15 to 80 days (reviewed [10]), similarly intermittent ovulations were observed in mares $\geq$20 years [23]. Lengthening of the follicular phase with consistently elevated concentrations of gonadotropins was associated with minimal follicular growth and correlated with impending reproductive senescence for individual mares [23].

The failure of follicular growth in the older mare and woman is probably caused by depletion of primordial follicles in the ovaries. A temporal association has not been studied between the number of ovarian follicles and age in the mare. However, a reduced population of follicles is supported by the reduced number of antral follicles that are imaged by ultrasound in the older mare. Old mares with elongated follicular phases had only 1.5 follicles per ovulatory wave, which was significantly less than mares with normal follicular phases [23]. One third of mares $\geq$20 years had only one follicle $\geq$20 mm during the spring transition when FSH concentrations would have been high [19]. In women aged 25–46 years, the number of small antral follicles imaged by ultrasound was correlated to reproductive status, and was postulated to reflect the size of the remaining pool of primordial follicles [24]. Histological evaluations of the ovaries from women 45–55 years was used to determine the effect of follicular depletion on the menopausal transition [17]. Researchers found that entry into perimenopause was associated with a 10-fold decline in the number of primordial follicles, and the follicular reserve was nearly exhausted with the approach of menopause.

The average age of onset of menopause for women in Western countries was approximately 50–51 years, but the age varied from 40 to 60 years [25]. Reproductive senescence appeared to occur in mares beginning at approximately 20 years. No ovarian activity was observed in 37% of horse mares over 24 years [26], and only 50% of pony mares $\geq$20 years had sequential ovulations, while 19% of the pony mares did not grow a follicle or ovulate during 60 days of monitoring [23]. Concentrations of gonadotropins were elevated after cessation of reproduction in women [27] and mares [23]. Because of individual variation in time to reproductive failure, definitive reproductive changes at specific ages in mares or women were not very meaningful. However, te Velde et al. [25] hypothesized

that the progression of reproductive events leading to the time of reproductive cessation was probably fixed.

In summary, reproductive cycles in mares and women had similar age-associated changes. The reproductive lifespan of women appeared to be approximately twice that of mares. Changes in FSH concentrations were detected after 30 years in women [12] and 15 years in mares [16] and were associated with a shortening of the follicular phase and cycle. With further aging, reproductive cycles became longer, and intermittent ovulations occurred. Ultimately, reproductive cycles ceased at approximately 50 years in women and 25 years in mares. With our ability to repeatedly monitor ovarian function and hormone concentrations, the mare provides a potential model to evaluate the intrinsic mechanisms associated with ovarian failure. Reproductive seasonality in the mare provides the potential for multiple models: (1) normal cyclic during the natural breeding season, (2) FSH stimulation with insufficient LH to induce ovulation during the spring transition, and (3) noncyclic during the winter months, with quiescence of the hypothalamic–pituitary–ovarian axis.

3. Follicular and oocyte maturation

The mare is poorly suited to maintaining more than one fetus within her uterus, and most mares ovulate a single follicle. During the follicular phase, mares can be induced to ovulate with human chorionic gonadotropin (hCG). Ovulation occurs approximately 36–37 h after hCG administration in mares [18] and in women [28], suggesting a comparable timeline for events associated with follicular and oocyte maturation. The mare may provide a unique perspective into events associated with in vivo maturation of the follicle and oocyte. The mare's long follicular phase, large follicle and timing from hCG to ovulation provide the potential to collect large amounts of follicular fluid or cells at precise time points and to inject or manipulate the follicle.

The effect of LH was historically thought to be on the cumulus-oocyte complex in most species, although oocytes and cumulus cells did not have measurable numbers of LH receptors [29]. More recent investigations in mice demonstrated that LH stimulates granulosa cells to produce the epidermal growth factor (EGF)-like growth factors, amphiregulin, epiregulin and betacellulin [30]. When release of these factors was impaired in the follicle enclosed oocytes of rats with a broad-spectrum matrix metalloprotease inhibitor, meiotic maturation induced by LH was suppressed [31]. Epidermal growth factor-like growth factors bind to EGF receptors [32] on cumulus cells and result in cumulus expansion and germinal vesicle breakdown. In mice, a transient increase in expression of EGF-like growth factor mRNAs occurred within 1 to 3 h after hCG administration and declined by 6 h [30]. Preliminary work in mares demonstrated increased mRNA for amphiregulin and epiregulin in granulosa cells from 0 to 2 h after hCG administration, with amphiregulin mRNA increasing again from 2 to 4 h after hCG, and remaining elevated at 6 h, the last collection time studied [33]. In a subsequent experiment in our laboratory, mRNA expression was examined in the granulosa cells of young and old mares at 3-h intervals after administration of equine recombinant LH. Expression of amphiregulin and epiregulin peaked at 9 h before declining to approximately baseline at 12 h, and different patterns of expression were observed for young versus old mares [34]. Lindbloom et al. [33] attempted to determine if phosphodiesterase (PDE) mRNA expression changed within the oocyte in response to hCG. Isoforms of PDE are found in cumulus cells (PDE 4D) and oocytes (PDE 3A) [35]. By 6 h after administration of hCG, a significant response was not observed using small numbers of samples. However, subsequent research demonstrated an increase in PDE3A in equine oocytes at 9 h after follicular stimulation with equine recombinant LH [34], suggesting a temporal association in the expression of mRNAs for EGF-like growth factors and PDE3A. Results of the studies indicated that equine follicles responded to hCG/LH in a similar fashion to murine follicles, with differences in timing. Because the interval from hCG to ovulation is similar in the mare and woman, the mare may provide a model with temporal similarities to the woman in the molecular events associated with development of the follicle, oocyte, and ovulation.

Due to size and accessibility via transvaginal procedures, the equine follicle has unique strengths for studying molecular events during in vivo maturation of the follicle and oocyte. The equine follicle was approximately 45 mm in diameter prior to ovulation, and the long follicular phase of the mare was associated with an average growth rate of 3 mm per day for the dominant follicle [18]. Compared with many other species, follicular growth in the mare is magnified and in slow motion. The follicle destined for ovulation can usually be identified approximately a week prior to ovulation, and because of its large size and early detection, follicular observations and manipulations are easily done.

In our laboratory, we are currently comparing the events associated with oocyte maturation in vivo

between young and old mares. The large, easily assessable equine follicle allowed us to collect [36] large numbers of follicular cells, follicular fluid and oocytes during specific stages of the cycle or specific times after follicular or systemic treatments. Preliminary research showed that changes in expression of mRNA for PDE3A in the oocytes of young and old mares occurred over a similar timeline; elevated PDE3A will ultimately result in reduced cAMP and initiation of oocyte maturation. However, the expression of mRNAs for amphiregulin and epiregulin in granulosa cells and for factors involved in oocyte-cumulus signaling (GDF9 and BMP15) and meiotic arrest (GPR3) were not synchronized in old and young mares, suggesting dissociation between oocyte maturation and ovulation with aging [34].

4. The effect of age on fertility and oocyte viability

A decline in fertility has been well documented in the older mare, beginning during the teen years. Madill [37] reviewed previous reports to determine reproductive indices in younger (2–11 years) and older mares (≥14 years). For the younger and older mares, respectively, the conception rates per cycle were 57 and 31%; foaling rates per cycle were 51 and 13%; and foaling rates per season were 82 and 48%. In a controlled experiment [38], young (5 to 7 years) and old (≥15 years) pony mares were inseminated using the same stallion and similar breeding management. The pregnancy rate on day 12 was significantly lower for old than young mares (32% versus 100%), and the embryo loss rate was significantly higher for old than young mares (62 and 11%). In woman, fecundity declines by 50% from 25 to 35 years (reviewed [39]). In Amish women that did not use birth control, the median age for the last birth was 38.5 years, with only 7% of births between 40 and 44 years, and <1% of births after 44 years; delivery rates after intrauterine inseminations were 13% for women 40 years of age, with no deliveries for women 43–47 years [40]). The age of women at last delivery was 10 years prior to the age at menopause, and te Velde et al. [25] speculated that the beginning of subfertility occurred about 10 years prior to the end of fertility, which preceded menopause by approximately 10 years. This same progression was noted in mares, with mares in their early teen years having regular cycles but reduced fertility. Equine fertility in the late teens to early twenties was markedly reduced, although many mares continued to have regular cycles. With continued aging, mares were infertile, and ovarian activity ceased.

5. Oocyte quality

Oocyte quality is ultimately the primary factor affecting reproductive performance in older mares and women, although causes and timing of the age-associated decline in oocyte quality have not been determined. Oocytes, donated from young women, were used to establish pregnancies in older women [41–43]. Sauer et al. [42] transferred embryos produced from women <35 years into the uteri of women up to 59 years; the establishment of pregnancy was not affected by age of the recipient, and recipient's age did not affect pregnancy outcome [44]. Results of the oocyte donation programs support reduced oocyte quality as the ultimate cause of reproductive failure in older women.

Findings were similar in mares when oocyte transfer procedures were used. In contrast to the clinical use of oocyte donations in women, where the older female wishes to establish and carry a pregnancy, oocyte transfer in mares has been used to establish pregnancies in young recipients using the oocytes from older, valuable mares. The initial work in this area was done to determine the effect of age on the viability of oocytes from young and old mares (6 to 10 and ≥20 years, respectively) [45]. Oocytes were collected from preovulatory follicles, and mature oocytes were transferred into the oviducts of young, inseminated recipients. Fertilization and embryo development occurred within the reproductive tracts of young mares. Early embryo development was determined by imaging embryonic vesicles within the uteri of recipient mares. Oocytes from young versus old mares resulted in significantly more embryonic vesicles (92% and 31%, respectively). This research confirmed that an age-associated decline in oocyte quality is a primary factor in reduced fertility in the old mare.

Morphological comparisons have been done for oocytes from young and old females. Oocytes from the preovulatory follicles of young and old mares (3–19 years and >19 years, respectively) were examined by light and electron microscopy. Significantly more oocytes from old than young mares contained large (>1% volume) vesicles, and the mean number of large vesicles per oocyte was greater for old than young mares [46]. In contrast to oocytes from young mares, some of the oocytes from old mares had morphological anomalies, including one with a vesicle occupying >50% of the ooplasm and one with a vesicle within the nucleus; other oocytes had atypical shapes, sections of ooplasm without organelles, and sections of oolemma with sparse microvilli. A significant increase in the ooplasmic fraction of vacuoles was also observed in

oocytes from advanced age (38–45 years) versus young (25–32 years) women after the evaluation of ovarian biopsies [47]. The increased vacuolization was postulated to be caused by the accumulation of damage in the oocytes [47].

The causes of morphologic defects within oocytes from aged females are not known. Mutations in mitochondrial DNA and production of reactive oxygen species have been postulated to have a detrimental effect on oocytes from older females [48–50] with the potential to compromise chromosomal segregation in the oocyte [51,52]. Mitochondrial damage has been reported in the oocytes from old mares. Oocytes were collected from the ovaries of mares (young, $\leq$11 years and aged $\geq$12 years) at an abattoir and matured in vitro. After maturation in vitro, oocytes from the aged mares were often swollen with extensively damaged cristae [53]. These findings confirm those of Thouas et al. [50] for mice, that the mitochondria in oocytes from aged females are more sensitive to experimentally induced damage. Mitochondrial and ooplasmic transfers have been postulated as a method to improve oocyte quality by providing a healthy infusion of mitochondria or ooplasm [54–56]. Most of the research in ooplasmic transfers has been conducted on rodent models, and its value for the oocytes from aged females has not been definitively determined. Research in this area has yet to be done in the mare, although the required techniques are available, and the procedure has potential clinical relevance.

The developmental potential of oocytes requires molecular and cellular properties that allow normal growth, maturation, and development after fertilization. This includes factors that are produced from the oocyte that are essential for the coordination of oocyte and follicular maturation and for initiation of primordial follicle growth [57]. Oocyte-specific genes have been identified that correspond to oocyte competence, and targeted deletions for essential communication genes in murine oocytes resulted in altered competence for developmental potential [58]. Age-related changes in global gene expression have been studied using microarray gene expression profiles to compare metaphase II oocytes from young (5–6 week) and old (42–45 week) mice. The most notable differences were in mitochondrial function, oxidative damages, and stress responses [59]. Although molecular and genetic studies have the potential to identify cellular differences associated with oocyte competency and aging, the limited availability of equine oocytes and microarrays could impair use of the mare for global gene expression profiles. However, the horse would provide an excellent model for further investigations of age-associated alterations in mRNA expression, after gene selection using other animal models.

6. Incorporation of an equine model

The equine model provides a method to incorporate whole-animal and molecular research. To date, the majority of molecular and genetic studies of reproductive aging used large numbers of oocytes and specific lines or genetically altered mice. For instance, Hamatani et al. [59] used three sets of 500 oocytes from young and old C57BL/6 mice for microarrays. The use of comparable models is invaluable for understanding reproductive aging; however, studies in humans and horses will probably necessitate smaller numbers and more population variance. Our current studies in mares include the use of individual follicles with mRNA expression determined per follicle and per oocyte [33,34]. Disadvantages of this approach are obvious, with the potential for error associated with small sample size and variability among mares. However, this approach allows the study of individuals. We anticipate more variability in endpoints for old than young mares, and although this may complicate analyses, a more accurate reflection of aging in women may be obtained. Because follicular characteristics and hormone profiles can be monitored in mares, we can use selected criteria to obtain mares in apparently different stages of aging and correlate whole-animal and molecular end points.

Clinical use of assisted reproduction procedures in horses provides insight into clinical applications in human reproduction. In contrast to many other domestic species, horses are often selected based on criteria other than fertility, and the value of offspring promotes use of assisted reproductive procedures. In the assisted reproduction program in our laboratory, most mares are older with fertility problems [60] or are young and donating oocytes for intracytoplasmic sperm injections. Consequently, research advances can be incorporated into the management of clinical cases.

7. Conclusions

The mare represents an excellent animal model in many respects. The reproductive tract of the mare is easily assessable for monitoring and manipulations, with many procedures causing minimal or no discomfort to the animal. Reproductive cycle characteristics in the mare are similar to women, cumulating in the ovulation of a single follicle after a long follicular

phase. Age-associated changes in fertility and cyclic activity in the mare parallel the woman, with ovarian failure the probable cause. The age-associated decline in fertility is correlated with reduced oocyte quality in both species, and the decline in oocyte viability could be the result of maintenance of oocytes in meiotic arrest for decades. The mare provides a model to integrate whole-animal and molecular research for the study of oocyte maturation in vivo and for the study of age-associated changes in the cycle, follicle and oocyte.

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