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Molecular and functional characteristics of dominant follicles during spring transition in mares: a review

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Summary

Many mares enter a period of spring transition between winter anoestrus and cyclicity when follicles may reach preovulatory size, yet fail to ovulate. The aim of the present study was to determine the morphological, functional and molecular characteristics of large dominant transitional follicles. Follicle growth was monitored regularly from February through July in 25 pony mares. In some of the mares, one of the ovaries was removed on the day after an anovulatory follicle reached 30 mm, and the contralateral ovary was removed during cyclicity on the day after the preovulatory follicle reached 30 mm. Samples of the large follicle were processed for immunohistochemistry and in situ hybridisation, and follicular fluid was frozen. Hormonal control of growth of the large transitional follicles was similar to that seen during cyclicity. However concentrations of oestradiol were significantly lower than during growth of the preovulatory follicle and LH remained low until ovulation. During growth of anovulatory follicles, uterine oedema was sometimes present along with low, but significant, elevations in oestradiol. Transitional follicles had poorly developed theca that received a scant blood supply and there was little VEGF, the main ovarian angiogenic factor, in these follicles. Transitional follicles contained only low concentrations of oestradiol and progesterone and there was low expression of mRNA encoding StAR, the steroidogenic enzymes, and LHr in the follicle walls. Our results show that the poor steroidogenic capacity of transitional follicles is directly related to low gene expression of the steroidogenic enzymes, possibly caused by the low levels of LHr and IGF mRNAs. The scant blood supply and poor development of the theca would also contribute to the steroidogenic incompetence of these follicles. Expression of IGF-I and –II mRNAs were lower in transitional anovulatory follicles than in preovulatory follicles and intrafollicular concentrations of IGFBP-2 were significantly higher in transitional than in preovulatory follicles. These results suggest that the bioavailability of intrafollicular IGF is enhanced in large preovulatory follicles during the breeding season and suggests that intrafollicular IGF bioavailability must exceed a threshold level before an ovulation can occur.

Keywords: follicles, transition, hormones, IGF, mare

Molekulare und funktionelle Charakterisierung der dominanten Follikel in den Übergangszyklen im Frühjahr bei Stuten - eine Übersicht

Viele Stuten zeigen im Frühjahr, zwischen dem Winteranöstrus und der zyklischen Aktivität, eine Übergangsperiode in der die Follikel zwar präovulatorische Größe erreichen, aber nicht ovulieren. Das Ziel der vorliegenden Studie war die morphologische, funktionelle und molekularbiologische Charakterisierung großer dominanter Follikel in der Übergangszeit. Dazu wurde bei 25 Ponystuten zwischen Februar und Juli das Follikelwachstum regelmäßig kontrolliert. Bei einigen der Stuten wurde ein Ovar am Tag nachdem ein anovulatorischer Follikel 30 mm groß war enfernt, das kontralaterale Ovar wurde während des Zyklus, einen Tag nach Erreichen eines 30 mm großen präovulatorischen Follikels entnommen. Proben der großen Follikel wurden immunhistochemisch und mittels inSitu-Hybridisierung beurteilt und die Follikelflüssigkeit wurde tiefgefroren. Die hormonelle Kontrolle des Wachstums der großen Follikel in der Übergangszeit entsprach der während des Zyklus mit einem Anstieg von FSH und Inhibin A im Zusammenhang mit dem Wachstum eines dominanten Follikels. Die Östradiolkonzentrationen waren hingegen signifikant niedriger als während der Entwicklung eines präovulatorischen Follikels und LH blieb bis zur Ovulation niedrig. Während des Wachstums eines anovulatorischen Follikels trat in einigen Fällen ein uterines Ödem zusammen mit einem geringen, aber signifikanten Anstieg von Östradiol, auf. Die Follikel in der Übergangsphase zeigten eine wenig ausgeprägte Theka mit schwacher Blutversorgung und wenig VEGF, dem hauptsächlichen ovariellen angiogenetischen Faktor in diesen Follikeln. Die Übergangsfollikel enthielten nur geringe Mengen an Östradiol und Progesteron und die mRNA von StAR, den steroidogenen Enzymen, und von LHr wurde in den Follikelwänden nur geringgradig nachgewiesen. Die Ergebnisse zeigen, daß die niedrige steroidogene Kapazität der Übergangsfollikel in direktem Zusammenhang mit der niedrigen Genexpression der steroidogenen Enzyme, möglicherweise aufgrund des niedrigen Niveaus an LH- und IGF-mRNA, steht. Die geringere Blutversorgung und die schwächer entwickelte Theka führen möglicherweise ebenfalls zu der steroidogenen Inkompetenz dieser Follikel. Die Expression der IGF-I und –II mRNA war in den anovulatorischen Übergangsfollikeln niedriger als in präovulatorischen Follikeln, und die intrafollikulären Konzentrationen von IGFBP-2 erwiesen sich aös significant höher in Übergangsfollikeln als in präovulatorischen Follikeln. Diese Ergebnisse lassen vermuten, dass die Bioverfügbarkeit des intrafollikulären IGF in den großen präovulatorischen Follikeln während der Zuchtsaison erhöht ist und eine Mindestkonzentration erreicht haben muss, bevor eine Ovulation statt finden kann.

Schlüsselwörter: Reproduktion, Follikel, Übergangszyklus, Hormone, IGF, Stute

Introduction

The mare is seasonally polyoestrus, with the natural breeding season extending from May to October (*Ginther* 1974). During winter anoestrus, the hypothalamo-pituitary axis is essentially non-functional. GnRH secretion is greatly reduced, possibly via dopaminergic inhibition (*Besognet* et al. 1996), and the pituitary fails to release significant amounts of gonadotrophins (*Hart* et al. 1984). Pituitary content of LH is low, but FSH content is unchanged. In response to the low con-

centrations of circulating gonadotrophins during winter anoestrus, mares have small, hard ovaries. In spring transition, follicles initially grow and regress, but do not exceed 35 mm in diameter (*Ginther* 1990). In many mares, waves of anovulatory follicle development then proceed, characterised by serial growth and regression of large (>38 mm) dominant follicles which are steroidogenically-incompetent. When a dominant oestrogenic follicle develops in the ovaries, the high circulating concentrations of oestradiol induce increased synthesis and secretion of GnRH and/or pituitary LH subunit mRNA expression (*Sharp* et al. 2001). This results in release of LH in sufficient amounts to cause ovulation (*Sharp* et al. 1991). Little was known in dominant transitional follicles of the factors thought to be critical to follicle maturation and ovulation: patterns of circulating FSH and inhibin A, steroidogenic enzymes, vascularity, morphology, and the IGF system.

Inhibin is thought to be important in control of FSH secretion and follicular growth in mares (*McCue* et al. 1992, *Nambo* et al. 1998). A number of studies have reported circulating concentrations of immunoreactive inhibin in cyclic mares (*Bergfelt* et al. 1991, *Nagamine* et al. 1998). These assays do not distinguish between the biologically active dimeric forms of inhibin and free monomeric a-subunits, which are thought not to be biologically active, and can antagonise the actions of FSH on granulosa cells by binding to FSH receptors (*Schneyer* et al. 1991). Immunoreactive inhibin and inhibin isoforms with pro- and -aC immunoreactivity, increase in late dioestrus in mares and peak on the day of ovulation (*Bergfelt* et al. 1991, *Nagaoka* et al. 1999). However no information was available on hormonal control of follicular development in transitional mares.

The steroidogenic potential of follicles depends upon the presence of gonadotrophin receptors, the availability of cholesterol, regulated by StAR, and the activity of rate-limiting steroidogenic enzymes. In order to investigate whether these were deficient in transitional follicles, levels of mRNA encoding the steroidogenic enzymes, StAR and LHr were compared in large follicles collected during spring transition and the breeding season using in situ hybridization, which gave information on both spatial distribution and levels of the respective mRNAs.
Blood vessel development serves a crucial role in follicular maturation (*Richards* 1980), and during follicle growth, a rich capillary plexus develops in the thecal layer surrounding the avascular granulosa cells. Work in primates has shown that the density of the microvascular network of follicles destined to ovulate is at least double that of follicles destined to become atretic (*Zeleznik* et al. 1981). This increased vascularity results in greater delivery of gonadotrophins to preovulatory follicles. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine stimulating blood vessel formation and enhancing microvascular permeability (*Dvorak* et al. 1995). Expression of VEGF mRNA and protein has been reported in follicles and corpora lutea of various species (*Barboni* et al. 2000, *Kashida* et al. 2001, *Ravindranath* et al. 1992). However, very little was known about the relative vascularity of equine ovulatory and anovulatory transitional follicles although it is clear that degree of vascularisation may be a critical factor in determining their subsequent fate.
Growth factors, in particular the insulin-like growth factor (IGF) system, are thought to play a key role in ovarian follicular growth and atresia. The IGFs have a variety of effects on follicular and luteal cells including stimulation of steroidogenesis, via increased availability of steroid precursors and upregulation of steroidogenic enzyme expression and activity. Follicular concentrations of IGF-I increase in large equine follicles (*Bridges* et al. 2002), whereas IGF-II concentrations are not different among different sizes of follicle. IGF binding proteins (IGFBPs) present within the ovary bind with IGFs, prolonging the half-life, but blocking the biological action of the IGFs (for review see *Armstrong* and *Webb* 1997). Follicular concentrations of IGFBP-2 decrease during follicular growth and increase during atresia in most species, including horses (*Gerard* and *Monget* 1998). We hypothesized, therefore, that the bioavailability of IGF would be lower during spring transition, when follicles grow to preovulatory size but fail to ovulate, contributing to failure of normal follicular steroidogenesis and development at this time.

This paper presents results from a series of studies designed to give a detailed insight into the structure and function of transitional anovulatory follicles in mares.

Materials and Methods

Twenty five mares of mixed breeding, weighing 250-450 kg and aged 3-20 years (mean ± SEM: 9 ± 1.6 years), were studied from the beginning of February through to their second or third ovulation of the breeding season. Ovarian activity was monitored three times weekly by transrectal ultrasonography until detection of the first 25 mm follicle on the ovaries, when examinations were performed daily until first ovulation. Blood samples were collected by jugular venipuncture into evacuated heparinised tubes three times weekly until follicles of 20 mm were present in the ovaries, when blood samples were collected daily until first ovulation. Examinations and blood sampling then resumed on day 14 after ovulation until second ovulation. The ovary containing the dominant follicle was removed from 14 of the mares during one of the anovulatory follicular waves in spring transition on the day after the leading follicle reached 30 mm in diameter. The contralateral ovary was removed at the second or third ovulation of the breeding season on the day after the dominant follicle reached 30 mm in diameter. After ovary removal, follicular fluid was aspirated from the largest and second largest follicles by needle puncture. A portion of follicle wall was fixed in 4% paraformaldehyde for 24 h until paraffin embedding. Histological sections were subsequently stained with haematoxylin and eosin.
Hormone assays, immunostaining, in situ hybridization, and Western ligand blots were carried out as described previously (Xu et al. 1995, *Armstrong* et al. 1998, *Nicholas* et al. 2002, *Watson* et al. 2002a,b).

Results

During spring transition there was an average of 3.7 ± 0.7 waves with dominant follicles of >30 mm, at intervals of 9.9 ± 0.8 days. In all transitional mares, each follicle wave was preceded by a significant elevation ($P < 0.001$) in FSH. The increase in FSH was observed when the follicle reached 15-22 mm. Two days later the follicles were 20-24 mm in diameter. However concentrations of FSH did fluctuate during

spring transition and some peaks were found that were not apparently associated with initiation of follicular growth.

Concentrations of plasma oestradiol consistently reached higher levels ($P < 0.01$) during the growth of an anovulatory follicle than in the three days before and after this time. Any peak in oestradiol concentrations was, however, short-lived and decreased as soon as the follicle started to regress. During the growth phase of an anovulatory follicle, concentrations of inhibin A and inhibin pro- and -aC-containing isoforms were significantly higher ($P < 0.05$) than in deep winter anoestrus. In spring transition there were negative correlations between plasma inhibin pro-aC isoforms and FSH ($P = 0.06$) and between plasma inhibin A and FSH ($P = 0.1$) which approached significance.

Mean peak concentrations of FSH at the time of emergence of the first preovulatory follicles were not significantly different to those measured at time of emergence of anovulatory follicles for the same mares in spring transition. The peak concentration was significantly higher ($P < 0.001$) than that measured 2 days later. At this time the diameter of the dominant follicle was 21-32 mm which was not significantly different from the growth rate of the anovulatory follicles. Over the 10 days prior to the first ovulation, circulating concentrations of oestradiol, inhibin A and inhibin pro-aC isoforms increased gradually, with oestradiol peaking 2 days before ovulation, and inhibin pro- and -aC isoforms peaking on day of ovulation.

In cyclic mares, mean concentration of oestradiol during growth of an ovulatory follicle was significantly higher ($P < 0.001$) than during the growth of an anovulatory follicle. Plasma concentrations of inhibin A were significantly higher ($P < 0.05$) during growth of a preovulatory follicle than during growth of an anovulatory follicle in spring transition (see *Watson* et al. 2002a). Concentrations of oestradiol, progesterone and inhibin A were significantly lower in fluid collected from dominant, anovulatory transitional follicles than from preovulatory follicles. Although inhibin pro- and -aC isoforms tended to decrease in preovulatory follicles compared with transitional anovulatory follicles, these differences were not significant (see *Watson* et al. 2002b).

Eleven mares that had serial large anovulatory follicles, had uterine oedema at some point prior to their first ovulatory oestrus of the breeding season. The oedema was first detected 16 to 86 days before first ovulation. In mares that did not have anovulatory follicle waves, there was no detectable uterine oedema prior to first ovulatory oestrus. During anovulatory follicle waves, oestradiol concentrations were higher ($P < 0.05$) on days when uterine oedema was observed than on consecutive days when uterine oedema was not present. Peak plasma oestradiol concentrations and mean oestradiol concentrations during growth of the dominant follicle > 25 mm were significantly higher ($P < 0.001$) during growth of follicles at first and second ovulation than during the growth of an anovulatory wave (see *Watson* et al. 2003a)

Histology

Both anovulatory and preovulatory follicles had a well organised layer of granulosa cells in contact with the basement membrane. The theca interna in preovulatory follicles comprised a thick layer of plump polyhedral cells with a pale nucleus and cytoplasm, whereas the theca layer of the anovulatory follicles was thin and poorly developed in most parts with only sparse foci of polyhedral cells (see *Watson* and *Al-zi'abi* 2002).

Vascularity

Immunostaining for VEGF was confined to the theca interna. In preovulatory follicles, the entire theca interna layer stained strongly and diffusely for VEGF. Immunostaining in the transitional follicles was scant with patchy staining in the thin theca layer. Immunostaining for von Willebrand Factor (vWF) was confined to endothelial cells of blood vessels in the theca interna. The theca was well supplied with blood vessels in the preovulatory follicles whereas the transitional follicles had a relatively avascular theca. A significantly greater ($P < 0.05$) area of tissue was stained for vWF in the preovulatory follicles than in the transitional follicles. Positive staining for Ki67 (proliferation) was confined to cell nuclei and was frequently present in the granulosa cells and thecal endothelial cells of preovulatory follicles. No staining was seen in endothelial cells of transitional follicles and only occasional granulosa cells were stained (see *Watson* and *Al-zi'abi* 2002).

Steroidogenic enzymes and LHr

There was significantly lower ($P < 0.001$) expression of StAR and steroidogenic enzymes in transitional follicles than in preovulatory follicles. Expression of LHr mRNA was lower in both granulosa ($P < 0.05$) and theca ($P < 0.001$) cells in transitional than preovulatory follicles (see *Watson* et al. 2003b).

IGF System

Levels of IGFBP-2 were significantly higher ($P < 0.05$) in fluid from transitional follicles than in fluid from preovulatory follicles. Expression of mRNAs encoding IGF-I and –II was significantly lower ($P < 0.001$) in transitional anovulatory follicles than in preovulatory follicles (*Bae* et al. 2003). In transitional follicles, IGFBP-2 mRNA expression was similar in theca and granulosa cells. There was a significant increase ($P < 0.001$) in thecal expression in preovulatory follicles compared with transitional follicles, whereas expression in granulosa remained unchanged.

Discussion

In these studies, during spring transition there was an average of 3.7 waves with leading follicles of >30 mm, at intervals of approximately 10 days. This pattern was very similar to that previously reported by workers in Florida and Wisconsin (*Davis* et al. 1987, *Ginther* 1990). Concentrations of FSH were low in mares in deep winter anoestrus when the ovaries were small and hard with minimal follicular activity, and increased by the time the mares had significant follicular activity on their ovaries. These findings are in agreement with previous reports (*Turner* et al. 1979, *Alexander* and *Irvine* 1991). We found that the emergence of every dominant follicle, anovulatory or ovulatory, was preceded by an FSH surge when the follicle was 15-23 mm in diameter, suggesting

that follicular wave emergence is stimulated by FSH regardless of season. This has been confirmed by a later paper (*Donadeu* and *Ginther* 2002). In the presence of anovulatory follicle waves in spring, circulating concentrations of inhibin A were higher than in anoestrus, but were significantly lower than during the same period of growth of an ovulatory follicle (*Watson* et al. 2002a). Furthermore, in contrast to preovulatory follicles which contained high concentrations of circulating inhibin A, large anovulatory transitional follicles in our mares contained significantly lower concentrations of inhibin A (*Watson* et al. 2002b). The absence of large follicles in deep anoestrus may explain the low circulating concentrations of the isoforms of inhibin measured in the present study. During winter anoestrus, greater variations were seen in FSH than during cyclicity, and so perhaps the absence of suppression by high circulating concentrations of inhibin A permitted intermittently elevated concentrations of FSH at this time.

We have shown in these studies, in agreement with *Davis* and *Sharp* (1991), that anovulatory follicles are not associated with high circulating concentrations of oestradiol. However, there was a clear significant elevation in concentrations of both oestradiol and inhibin A during anovulatory waves compared with periods when no large follicles were present. Furthermore, we showed a significant decrease in FSH as the dominant follicle grew after emergence. Therefore, we would suggest that the combined effect of inhibin and oestradiol, which is more strongly inhibitory than either hormone on its own (*Miller* et al. 1981), may be important in suppressing the pituitary release of FSH that was measured during spring transition when the dominant anovulatory follicle reached a mean of 22 mm in diameter. The similarity in diameter and in hormonal patterns between dominant spring transition and during the breeding season strongly suggests that these large anovulatory follicles show dominance in a similar manner to follicles during the oestrous cycle. In the present studies, concentrations of inhibin A were higher in preovulatory follicles than in either transitional or subordinate follicles. As the fate of both transitional and subordinate follicles in mares is regression, it is possible that the elevated concentrations of inhibin A in the preovulatory follicles of mares are anti-atretogenic. Alternatively inhibin A may be involved in the process of ovulation.

The small elevations in oestradiol concentrations during the growth of anovulatory follicle waves, were sometimes associated with uterine oedema. It seems likely therefore that in the absence of progesterone, the mare is highly sensitive to very low concentrations of oestrogen during spring transition at the level of both the brain and the uterus. The presence of oestrous-like uterine oedema during anovulatory waves means that oedema cannot be used as definite proof that a large follicle is destined to ovulate (*Watson* et al. 2003a).

Poor vascularity, characterised by visibly pale follicular walls, has been reported to be a sign of atresia in equine follicles (*Kenney* et al. 1979). In the present study, the lining of transitional follicles was paler than that of preovulatory follicles. This observation was confirmed by immunostaining for vWF which identifies endothelial cells. There was a significantly smaller area of vWF immunostaining in the theca of transitional than preovulatory follicles. The results of the present study therefore indicate increased vascularity in the preovulatory follicles compared with transitional follicles (*Watson* and *Alzi'abi* 2002). Furthermore, the transitional follicles contained very little VEGF, which is important in promoting angiogenesis, whereas VEGF appeared to be abundant in the preovulatory follicles. VEGF also has the ability to increase the permeability of the microvasculature (*Murohara* et al. 1998). Thus in preovulatory follicles, the richer blood supply, in combination with increased permeability of blood vessels, will allow increased provision of oxygen, nutrients and substrates, as well as circulating gonadotrophins, which are essential for follicular health, growth, and steroidogenesis.

The growth of new blood vessels can also be monitored by measuring endothelial cell proliferation (*Rodger* et al. 1997). Cells with the morphological appearance of endothelial cells that stained positively for Ki67 were present only in the theca of preovulatory follicles, suggesting active proliferation of blood vessels in these follicles. Many of the granulosa cells in these follicles were also positively stained, whereas staining was infrequent in the granulosa of the transitional follicles indicating active cell division in the preovulatory follicles in contrast to the transitional follicles.

As follicle health and growth is dependent on gonadotrophin stimulation, it is likely that the low circulating concentrations of LH failed to stimulate adequate production of angiogenic factors, including VEGF. The low levels of angiogenic factors then led to poor thecal vascularisation and vascular permeability, both of which are essential for trophic support of the actively dividing follicular cells. Poor vascularity in turn will contribute to inadequate delivery of gonadotrophins and other trophic factors to the follicle to sustain development. In these mares therefore, the low levels of thecal VEGF could result in failure of further development and subsequent atresia of transitional follicles.

The low levels of mRNAs for steroidogenic enzymes found in transitional follicles in these studies, indicate that oestrogen production in transitional follicles is compromised by both a reduction in the supply of aromatizable substrate in thecal tissue and reduced aromatase activity in granulosa cells. At the ovarian level, it has been shown that the acquisition of steroidogenic competence by a large dominant follicle precedes initiation of cyclicity in transitional mares by approximately 5.5 days (*Sharp* et al. 2001). These authors suggested that increases in oestradiol concentrations may be the key factor in stimulating release of LH prior to first ovulation. In larger follicles, expression of LHr and LH binding increase (*Fay* and *Douglas* 1987, *Goudet* et al. 1999). This is accompanied by an increase in steroidogenic enzymes and concentrations of oestradiol in follicular fluid (*Watson* and *Thomson* 1996, *Belin* et al. 2000). In the present study, mRNA encoding LHr was low in transitional follicles. Final follicular growth and development in the mare is dependent on support by LH (*Gastal* et al.1999, *Watson* et al. 2000). It is likely therefore that both the low circulating concentrations of LH and low expression of follicular LHr in transitional mares contributed to the deficiency in mRNAs for the steroidogenic enzymes.

Intrafollicular concentrations of IGF-I, but not IGF-II, have been positively correlated with follicle size in mares (*Spicer* et al. 1991, *Bridges* et al. 2002) and concentrations are higher

in follicles that assume dominance (*Ginther* et al. 2002). IGF-I stimulates mitogenesis, steroidogenesis and upregulates gonadotrophin receptors (*Armstrong* and *Webb* 1997). In other domestic species, changes in IGFBP concentrations are more closely associated with follicle growth and regression than with changes in levels of IGF-I and –II (*Spicer* and *Echternkamp* 1995), because only free IGF has biological activity. In mares, intrafollicular levels of IGFBP-2, -4, and -5 decrease in large, oestrogenically-active dominant follicles compared with subordinate follicles or large regressing follicles which contain only low concentrations of oestrogen (*Gerard* and *Monget* 1998, *Bridges* et al. 2002). Therefore it has been proposed that follicular levels of these binding proteins are closely related to the physiological status of equine follicles. Although there was a significant increase in IGFBP-2 mRNA in theca of preovulatory follicles, concentrations of IGFBP-2 were lower in preovulatory follicles than in transitional follicles. One explanation of the discrepancy between message and protein would be the presence of IGFBP-2 protease activity in follicular fluid of preovulatory follicles. However in equine follicular fluid it is thought that there is little or no proteolysis of IGFBP-2 (*Bridges* et al. 2002). The high levels of IGFBP-2 in transitional follicles, by lowering free IGF concentrations, may contribute to the steroidogenic inadequacy of these follicles. Reduced availability of IGF may also contribute to the low LH receptor mRNA expression (*Magoffin* and *Weitsman* 1994; *Watson* et al. 2003b), and the low levels of VEGF and proliferation in dominant transitional follicles (*Watson* and *Al-zi'abi* 2002, *Martinez-Chequer* et al. 2003).

We conclude that the poor steroidogenic capacity of transitional follicles is directly related to low gene expression of the steroidogenic enzymes, possibly caused by the low levels of LHr and bioavailability of IGF. The low levels of VEGF in transitional follicles are likely to have resulted in the scant blood supply and poor development of the theca which would also contribute to the steroidogenic incompetence of these follicles.

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