Plasma FSH, inhibin A and inhibin isoforms containing pro- and -αC during winter anoestrus, spring transition and the breeding season in mares

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Ten mares were studied from February (winter anoestrus) to their second ovulation in the breeding season to investigate the relationship between resumption of ovarian cyclicity in the spring and circulating concentrations of FSH, inhibin A and inhibin isoforms containing pro- and -αC immunoreactivity. An additional four mares were studied during one oestrous cycle. Growth and regression of ovarian follicles were monitored by transrectal ultrasonography. The frequency of blood sampling varied from three times a week to once a day, depending on the follicular activity present. Concentrations of FSH, oestradiol, inhibin A and pro- and -αC isoforms were low during deep winter anoestrus when minimal follicular activity was present in the ovaries. During spring transition, an increase in FSH concentration preceded the emergence of each follicular wave. Concentrations of inhibins were significantly higher (P < 0.05) during growth of anovulatory follicles in spring transition than during winter anoestrus. Plasma concentrations of oestradiol and inhibin A were significantly higher (P < 0.001, P < 0.05, respectively) during the growth of preovulatory follicles than during the growth of transitional anovulatory follicles, but concentrations of inhibin pro-αC isoforms did not differ between the two types of follicle. During the oestrous cycle, there was a significant inverse relationship (P < 0.001) between concentrations of FSH and the inhibins. Plasma inhibin pro-αC isoforms, but not inhibin A, reached a peak on the day of ovulation. The results strongly indicate that FSH regulates growth of spring anovulatory and preovulatory follicles. Inhibins are likely to contribute to negative feedback on the release of FSH from the pituitary gland both during the transitional period and the breeding season in mares.

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 the FSH content remains unchanged and there appear to be differences in the proportion and type of pituitary gonadotrophs during anoestrus (Eagle and Tortonese, 2000). In response to the low concentrations of circulating gonadotrophins during winter anoestrus, mares have small, hard ovaries with only one or two follicles with a diameter of < 15 mm.

The period of spring transition is characterized by a resurgence of follicular activity, irregular oestrous behaviour, and resumption of secretion of gonadotrophins and ovarian steroids. Follicles initially grow and regress, but do not exceed 35 mm in diameter (Ginther, 1990). In many mares, waves of anovulatory follicular development proceed, which are characterized by rhythmic growth and regression of large (> 38 mm) dominant follicles. However, there is individual variation among mares, and some mares do not show clear anovulatory follicular waves (Ginther, 1990). The mean total duration from first detection of follicular growth to first ovulation is approximately 60 days (Sharp and Davis, 1993). It has been suggested that the acquisition of steroidogenic competence by follicles is the key to initiation of cyclicity in transitional mares (Sharp and Davis, 1993), and fluid collected from follicles in spring transition contains very low concentrations of oestradiol.

Inhibin is thought to be important in the control of FSH secretion and follicular growth in mares. Immunization against inhibin suppresses plasma concentrations of FSH, and increases both the number of growing follicles and ovulation rates (McCue et al., 1992; McKinnon et al., 1992; Nambo et al., 1998). Inhibins are dimeric proteins comprising one α-subunit and one of two β-subunits (βA or βB) forming...
two isoforms, inhibin A (α-βA) and inhibin B (α-βB). In addition, high concentrations of a precursor form of the α-subunit, measured as inhibin isoforms containing pro- and -αC immunoreactivity, have been identified in equine plasma (Nagaoka et al., 1999).

Most studies in normal cyclic mares have reported circulating concentrations of inhibin immunoreactive inhibin (Bergfelt et al., 1991; Roser et al., 1994; Nagamine et al., 1998). These assays do not distinguish between the biologically active dimeric forms of inhibin and free monomeric α-subunits, which are thought not to be biologically active. The α-subunits may be present in very high concentrations in the circulation, effectively masking the bioactive dimeric forms (McNeilly et al., 1994) and hence also our understanding of the role of the bioactive dimeric inhibin forms. The concentrations of immunoreactive inhibin and inhibin isoforms with pro- and -αC immunoreactivity increase in late dioestrous mares and reach a peak on the day of ovulation (Bergfelt et al., 1991; Roser et al., 1994; Nagamine et al., 1998; Nagaoka et al., 1999). Immunohistochemistry studies in the equine ovary indicate that dimeric inhibin A is secreted mainly by the granulosa and theca cells of large follicles, whereas the granulosa cells of small follicles are thought to secrete inhibin α-subunit (Nagamine et al., 1998).

Inhibins play an important role in folliculogenesis in the mare, but whether changes in concentrations of inhibin are involved in the initiation of ovarian cyclicity in the spring is unknown. The present study is the first to report the presence of inhibin A during the equine oestrous cycle, and the relationship between follicular growth and plasma FSH, inhibin A, inhibin isoforms containing pro- and -αC immunoreactivity, and oestradiol throughout the spring transition and into the breeding season.

**Materials and Methods**

Ten mares of mixed breeding, weighing 300–450 kg and aged between 3 and 10 years, each day during a complete oestrous cycle. The study was performed under the approval of the University of Edinburgh Ethics Committee and project licence obtained under the Home Office Animals (Scientific Procedures) Act 1986.

**Hormone assays**

Plasma FSH was measured by radioimmunoassay. A highly purified equine FSH (AFP-50228) was used for standards and iodination. The antiserum (AFP-2062096) was specific for equine FSH and used at a final dilution of 1:100000. Otherwise, the method was the same as that described by Watson et al. (2000). The main cross-reactivity of this antiserum was with equine LH (eLH; 2.5%). Cross-reactivity with all other hormones tested (eTSH, ePRL, eGH, eCG, hCG) was < 1%. Sensitivity of the assay was 0.5 ng ml−1. Intra- and interassay coefficients of variation were 6.3 and 10.2%, respectively. Displacement curves produced by serial dilutions of plasma and the addition of known amounts of FSH to samples containing low concentrations of FSH were parallel to the standard curve.

Concentrations of inhibin A and inhibin isoforms containing pro- and -αC immunoreactivity were measured using two-site ELISAs as described previously (Groome et al., 1994, 1995; Menon et al., 2000; Riley et al., 2000; Bleach et al., 2001), with some minor modifications to the inhibin A assay. In the inhibin A assay, 8% (w/v) SDS was used, and both the antibody (PPG 14/6) and streptavidin–alkaline phosphatase conjugate were diluted in casein buffer (Eurogenetics Ltd, Hampton). Standards used for inhibin A were purified dilutions of 32 kDa bovine inhibin A and for inhibin pro-αC immunoreactivity, a highly immuno-purified preparation from human follicular fluid was used. The slopes of the dilution curves with equine plasma were parallel to the standard curve. The intra- and interassay coefficients of variation were 6–7% for inhibin A, and 7.1 and 8.2%, respectively, for inhibin pro-αC. The assay sensitivities were 15 pg ml−1 for inhibin A and 6 pg ml−1 for inhibin pro-αC.

Plasma oestradiol concentrations were measured as described by Watson et al. (2000). Only plasma collected from transitional mares was assayed for oestradiol. Assay sensitivity was 2 pg ml−1. Intra- and interassay coefficients of variation were 4.6 and 7.8%, respectively.

**Statistical analyses**

Mean concentrations of FSH were calculated in plasma samples collected from mares in deep winter anoestrus.
throughout February (n = 4 mares) and from mares in early transition, before the onset of anovulatory follicular waves (n = 9 mares). Differences were compared using the Mann–Whitney U test. After the onset of anovulatory follicular waves, the highest concentration of FSH measured at the time of wave emergence (at 15–20 mm) was used to compare the differences in one of the anovulatory and ovulatory follicular waves in each mare (n = 10) by paired t test. Peak FSH concentrations at the time of follicular wave emergence in spring transition were compared with concentrations measured 2 days later by the paired t test. Data were log transformed before analysis. Follicle sizes were compared using the Student’s t test.

Mean concentrations of oestradiol during an anovulatory and ovulatory follicular wave were calculated from concentrations obtained each day when a follicle was between 25 and 40 mm in diameter. During spring transition, concentrations were compared during the growth phase (> 25 mm), and during the 3 days before and after follicular growth. Differences were analysed using the paired t test on log-transformed data.

Mean concentrations of circulating inhibin were calculated for each mare in samples collected once or twice each week in winter anoestrus, before the onset of follicular waves (7–12 samples from nine mares). Because of the small number of samples, it was not possible to compare deep winter anoestrus with early transition. Mean concentrations were also calculated from samples collected each day throughout one anovulatory follicular wave in spring transition, when the mares had an ovarian follicle of 25–40 mm in diameter (n = 8 mares) and throughout one ovulatory wave during cyclicity (n = 12 mares). Differences in hormone concentrations between groups were examined first by one-way ANOVA to determine whether there were overall significant differences (P < 0.05) between the groups. If a significant difference was noted, the Mann–Whitney U test was used. Mean concentrations of inhibin isoforms in oestrus and dioestrus were compared in the four cyclic mares using the paired t test. The relationships between inhibin A, inhibin pro-αC and FSH were investigated by Pearson’s correlation. Growth and regression of follicles were analysed by regression analysis.

**Results**

All mares were in seasonal anoestrus at the start of the study. Only four of the ten mares were classed as being in deep winter anoestrus, and these mares remained in this category throughout February. At the start of the trial, one mare had an anovulatory follicle of > 30 mm in diameter on one ovary, but this mare was not cyclic. Therefore, data were available for nine mares in the period of erratic follicular growth (early transition) between deep winter anoestrus and spring transition when large anovulatory follicles were present. One mare failed to have at least one anovulatory follicular wave before ovulation; therefore, data were included for spring transition from a total of nine mares.
In spring transition there were negative correlations between plasma inhibin pro-αC isoforms and FSH ($P = 0.06$) and between plasma inhibin A and FSH ($P = 0.1$), which were almost significant.

The size of the largest follicle increased steadily before the first ovulation, and had an increased rate of growth in the final 10 days (Fig. 3a). The number of other large follicles (> 15 mm) decreased rapidly in the 10 days before the first ovulation (data not shown). From day 8 before the first ovulation, the growth of the largest follicle increased linearly ($y = 33.3 + 1.25x; R^2 = 92.6\%; P < 0.001$), and the second largest follicle regressed ($y = 31.4 – 1.3x; R^2 = 73.2\%; P < 0.01$). There was an apparent decrease in the mean diameter of the largest follicle between day 10 and day 8 before ovulation because four of the mares had a large regressing anovulatory follicle at the time. The rate of growth of the preovulatory follicle was lower at the first ovulation than at subsequent ovulations ($y = 23.5 + 2.6x; R^2 = 96.3\%; P < 0.001; n = 9$ mares; Fig. 4b).

Mean peak concentrations of FSH at the time of emergence (15–23 mm in diameter; mean = 19.4 ± 1.3 mm) of the first preovulatory follicles (6.6 ± 0.5 ng ml$^{-1}$) were not significantly different from those measured at the time of emergence of anovulatory follicles for the same mares in spring transition. The peak concentration of FSH was significantly higher ($P < 0.001$) than that measured 2 days later (3.9 ± 0.4 ng ml$^{-1}$). At this time, the diameter of the dominant follicle was 21–32 mm (mean = 24.9 ± 1.5 mm), which was not significantly different from the growth rate of the anovulatory follicles. Over the 10 days before the first ovulation, circulating concentrations of oestradiol, inhibin A and inhibin pro-αC isoforms increased gradually: oestradiol reached a peak 2 days before ovulation (Fig. 3b), and inhibin pro- and αC isoforms reached a peak on the day of ovulation (Fig. 3c).

In cyclic mares, the mean concentration of oestradiol during growth of an ovulatory follicle (19.2 ± 1.8 pg ml$^{-1}$)
was significantly higher \( (P< 0.001) \) than that during the growth of an anovulatory follicle. Mean concentrations of inhibin pro- and \(-\alpha C\) isoforms were highest on the day of ovulation (Fig. 1). Plasma concentrations of inhibin A were significantly higher \( (P< 0.05) \) during the growth of an anovulatory follicle. Mean concentrations of immunoreactive inhibin A in mares and inhibin pro- and \(-\alpha C\) containing isoforms \((\text{O})\), and (b) plasma concentrations of FSH \((\text{A})\) and follicular growth \((\triangle)\).

In the present study, there was an average of 3.7 follicular waves with leading follicles of > 30 mm in diameter, at intervals of approximately 10 days during spring transition. This pattern was very similar to that reported by Ginther (1990) and Davis et al. (1987). The time of selection and divergence of the future preovulatory follicle, and the decline in the size and the numbers of subordinate follicles in the 8 days before the first ovulation in the present study is agreement with reports by Ginther (1990) and Turner et al. (1979). The faster growth rate of the preovulatory follicle at subsequent ovulations compared with the first ovulation has been reported (Ginther, 1990) and is probably due to the higher circulating LH concentrations at subsequent ovulations (Freedman et al., 1979). Circulating LH is thought to be important in the final growth and maturation phase of the follicle in mares (Gastal et al., 2000; Watson et al., 2000).

Concentrations of FSH were low in mares in deep winter anoestrus when the ovaries were small and hard with minimal follicular activity, whereas there was an increase in FSH by the time the mares had significant follicular activity on their ovaries. These findings are in agreement with reports by Turner et al. (1979) and Alexander and Irvine (1991). Silvia et al. (1987) showed that although concentrations of FSH were lower during deep winter anoestrus, the response to GnRH was greater during deep winter anoestrus than during spring transition or in cyclic mares. These authors concluded that during winter anoestrus, secretion of FSH was low because of reduced secretion of GnRH. In the present study, the emergence of all dominant follicles, anovulatory or ovulatory, was preceded by an FSH surge when the follicle was 15–23 mm in diameter. This finding is similar to that reported during the oestrous cycle by Palmer (1987) and Bergfelt and Ginther (1993). The requirement for FSH in follicle recruitment during the ovulatory season has been reported by Bergfelt and Ginther (1985) and Pineda et al. (1973), but the present study is the first to report increases in FSH before each anovulatory wave. The FSH surge in dioestrus reflects higher amplitude FSH pulses (Irvine et al., 1998), and ovulation tended to follow this period of high pulse amplitude within 10 days. More frequent sampling would have been necessary for a detailed profile of FSH concentrations, but from the results of the present study, it seems likely that recruitment is similar in both the spring transition and during the breeding season.

The present study is the first report of the presence of circulating concentrations of inhibin A in mares and inhibit concentrations in the period from winter anoestrus to cyclicity. Previous studies have reported circulating concentrations of immunoreactive inhibin in cyclic mares (Bergfelt et al., 1991; Roser et al., 1994; Nagamine et al., 1998; Nagaoka et al., 1999). The assays used in the present study detect forms of monomeric inhibin (\(\alpha\) inhibin) using the inhibit pro- and \(-\alpha C\) assay, as well as dimeric inhibin A. Only dimeric inhibin A, not the free \(\alpha\)-subunit, is biologically active in other species (Robertson et al., 1986; Knight

**Fig. 4.** The normal oestrous cycle of the mare. Each point represents the mean values (±SEM) of 4–14 mares. (a) Plasma concentrations of inhibin A (○) and inhibin pro- and \(-\alpha C\) containing isoforms (●), and (b) plasma concentrations of FSH (▲) and follicular growth (△).
et al., 1989). The inhibin $\beta_A$ subunit is confined to granulosa and theca cells in large equine follicles (Nagamine et al., 1998), and fluid collected from small follicles contained very low concentrations of both inhibin A and inhibin pro-$\alpha$C immunoreactivity (Tanaka et al., 2000). The absence of large follicles in deep anoestrus may explain the low circulating concentrations of the isoforms of inhibin measured in the present study. Furthermore, in contrast to preovulatory follicles, which produced high concentrations of circulating inhibins, large anovulatory transitional follicles in the mares used in the present study appeared to be producing significantly lower concentrations of dimeric inhibin.

Circulating concentrations of inhibin A and inhibin pro-$\alpha$C isoforms were higher in oestrus than in dioestrus, but only inhibin pro-$\alpha$C isoforms increased on the day of ovulation. Studies using a non-selective assay showed that plasma concentrations of immunoreactive inhibin were higher in oestrus than in dioestrus and were at a maximum on the day of ovulation (Bergfelt et al., 1991; Roser et al., 1994; Nagaoka et al., 1999). The peak in circulating concentrations of pro-$\alpha$C inhibin isoforms may originate from peritoneal absorption of follicular fluid at ovulation (Bergfelt et al., 1991). The present study showed that inhibin A did not increase on the day of ovulation compared with other days during oestrus. Concentrations of inhibin pro-$\alpha$C isoforms in follicular fluid of large follicles were approximately 100 times higher than those of inhibin A (Tanaka et al., 2000), and this may have been reflected in the circulating concentrations after ovulation. Therefore, it appears that the measured circulating increase in immunoreactive inhibin at ovulation derives predominantly from the $\alpha$-subunit rather than from dimeric inhibin.

FSH concentrations decrease approximately 8 days before ovulation both in the normal oestrous cycle (Miller et al., 1980; Bergfelt and Ginther, 1993) and before the first ovulation of the breeding season (Freedman et al., 1979; Alexander and Irvine, 1991). In the present study, it was shown that this is the time when circulating concentrations of biologically active dimeric inhibin are increasing, before the first and subsequent ovulations. Furthermore, in previous years in our cyclic pony mares, the largest subordinate follicle did not start to decrease in size until approximately 3 days before ovulation (Pedersen, 2000). This is at a time when circulating concentrations of inhibin A are very high and, therefore, the preovulatory increase in inhibin is temporally associated with regression of the subordinate follicle. Release of FSH does not seem to be closely regulated by GnRH in the breeding season or in winter anoestrus mares (Garza et al., 1986; Silvia et al., 1987). It has been suggested that both during anovulatory follicular waves in spring transition and during growth of the pre-ovulatory follicle, the dominant follicle produces suppressive substances that inhibit the growth of the smaller follicles by negative feedback on FSH at the pituitary. It is thought that these substances may be oestradiol or inhibin (Silvia et al., 1987; Ginther, 1990; Bergfelt and Ginther, 1993). During winter anoestrus, plasma concentrations of FSH, inhibin A and inhibin pro- and -$\alpha$C isoforms were low. Furthermore, in the presence of anovulatory follicular waves in the spring, concentrations of inhibin A were higher than at anoestrus, but were significantly lower than during the same period of growth of ovulatory follicles. 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 high concentrations of FSH at this time. Furthermore, it is possible that lack of strong negative inhibin feedback at this time may have permitted the higher gonadotrophin response to treatment with GnRH in anoestrus mares compared with that in cyclic mares reported by Silvia et al. (1987). It is notable that as transition progressed, the FSH response to GnRH decreased. This corresponds to the increasing concentrations of inhibin measured between anoestrus and cyclicity in the present study.

The present study, in agreement with Davis and Sharp (1991), has shown that anovulatory follicles are not associated with high circulating concentrations of oestradiol. However, there was a significant increase in concentrations of both oestradiol and dimeric inhibin during the anovulatory follicular waves compared with periods when no large follicles were present. Furthermore, there was a significant decrease in FSH as the dominant follicle grew after emergence. Therefore, 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 diameter of 22 mm. Donadeu et al. (2001) proposed that equine follicles acquire the ability to secrete inhibin with FSH-suppressing capacity by the time they reach 13 mm in diameter. However, the contributory effect of other follicular products cannot be discounted.

The preovulatory increase in inhibin in the present study corresponded well to the emergence of the preovulatory follicle at the time when other follicles regressed. Furthermore, there was a strong negative correlation between inhibin and FSH concentrations during the oestrous cycle in the present study as previously reported using a non-selective assay (Bergfelt et al., 1991; Nagaoka et al., 1999). Oestradiol also has a negative feedback effect on FSH in the mare and reduces the pituitary response to GnRH (Sharp et al., 1991). As in the transitional mare, it is likely that both inhibin and oestradiol contribute to the decrease in FSH. Furthermore, oestradiol has been suggested as a candidate for involvement in the mechanism that leads to deviation in the diameters of follicles in mares (Gastal et al., 1999). It is also possible that inhibin may play a role in deviation, as immunoreactive inhibin increases as early as 9 days before ovulation (Nagamine et al., 1998) or between day 7 and day 12 of the oestrous cycle (Bergfelt et al., 1991). However, inhibins are not thought to play a role in follicle dominance in cattle (Mihm et al., 2000). The role of inhibins in follicle selection in mares requires further study.

In conclusion, it appears that both inhibins and FSH have
an important role in controlling follicle growth during the spring transition as well as during the oestrous cycle in mares. The inverse relationship between FSH and inhibins strongly indicated a negative feedback effect of inhibins on pituitary release of FSH, both during the spring transition and cyclicity. The peak in plasma concentrations of immunoreactive inhibin on the day of ovulation appears to derive from follicular release of inhibin α-subunit isoforms rather than from dimeric inhibin A.

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