Seasonal effects on the response of ovarian follicles to IGF1 in mares

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Abstract

The response of follicles to IGF1 was compared between the transition into the ovulatory season (transitional period) and the ovulatory season (ovulatory period) in eight mares using a cross-over experimental design within periods. Granulosa cells were collected from follicles 15–24 or 25–34 mm and expression of *IGF1R*, *IGF2R*, *FSHR*, *LHCGR* and *PAPPA* was determined by qPCR. In addition, 10 mg IGF1 or vehicle were injected into the largest follicle (transitional period) or the second largest follicle (ovulatory period) of a follicular wave before the beginning of diameter deviation between the two largest follicles (mean diameters at injection 19.2 and 20.0 mm during transitional and ovulatory periods respectively). Follicular fluid was collected 24 h after injection for determination of free IGF1, IGFBP, inhibin A and oestradiol levels. Granulosa cells from follicles 25–34 mm, but not follicles 15–24 mm, expressed higher levels of *IGF1R* ($P=0.01$), *FSHR* ($P<0.007$) and *LHCGR* ($P=0.09$) during the ovulatory period than during the transitional period, whereas *IGF2R* expression was higher in transitional than ovulatory follicles ($P=0.06$). Follicular IGFBP2 levels were not different ($P>0.1$) between periods and treatments, whereas IGFBP5 levels were higher ($P<0.05$) during the ovulatory period. Finally, IGF1 injection before the beginning of deviation induced an approximately twofold increase ($P=0.01$) in follicular inhibin A levels during each period and did not affect oestradiol ($P>0.1$). These results suggest that, as during ovulatory waves, equine follicles during transitional waves are responsive to IGF1 before the beginning of deviation and that, therefore, inadequate IGF1 responsiveness before deviation may not underlie the deficient development of dominant follicles during transition.

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Introduction

The period of spring transition between the anovulatory season and the ovulatory season in the mare is characterised by renewed follicular growth (reviewed in Donadeu & Watson 2007). During this period, follicular waves occur that can sequentially produce several large, anovulatory follicles usually in association with irregular periods of oestrus. This makes reproductive management of these mares difficult as it is virtually impossible to predict with accuracy when ovulation will occur.

Similar to follicular waves during the ovulatory season, waves during transition are characterised by a deviation in diameter between the largest follicle and smaller follicles when the largest follicle reaches about 23 mm (Donadeu & Ginther 2004). The largest follicle thus continues growing and becomes dominant whereas smaller follicles (subordinate follicles) cease growing and regress. However, although transitional dominant follicles can reach final diameters similar to their ovulatory counterparts, they have an underdeveloped theca, are poorly vascularised, express low levels of receptors for luteinising hormone (*LHCGR*) and have

reduced capacity to produce steroids (Watson & Al-zabi 2002, Acosta *et al.* 2004, Watson *et al.* 2004b). These features critically determine the anovulatory fate of these follicles. For example, adequate vascularisation as well as high intrafollicular levels of oestrogen and other growth factors enhance follicular responsiveness to gonadotrophins, and this is essential to meet the increased trophic demands for the development of ovulatory-competent follicles (Ginther *et al.* 2003). In addition, an increase in the ability of dominant follicles to produce oestrogen at the end of the spring transition is believed to be critical for the occurrence of the LH surge that triggers the onset of the ovulatory season (Sharp *et al.* 1991, 2001).

The reasons for the morphological, biochemical and functional deficiencies of dominant follicles during the transitional period are not clear. Low circulating LH levels and low follicular expression of LH receptor likely play a role (Donadeu & Ginther 2002a, Watson *et al.* 2004b). In addition, follicular responsiveness to circulating follicle-stimulating hormone (FSH) may be altered during the anovulatory season (Donadeu & Ginther 2002a, 2003, King *et al.* 2008), although seasonal

changes in FSH receptor (*FSHR*) expression in follicles have not been determined. Another trophic factor that is likely involved is insulin-like growth factor 1 (IGF1). In ovulatory mares, IGF1 availability increases specifically in the dominant follicle before the beginning of diameter deviation (Donadeu & Ginther 2002b, Spicer *et al.* 2005) where IGF1 binds to IGF receptor type 1 (IGF1R) to amplify the effects of gonadotrophins on follicular cell proliferation and steroid production, among other functions (Glister *et al.* 2001). Expression of *IGF1R* has been reported to change during antral follicle growth in sheep (Perks *et al.* 1995) whereas changes in *IGF1R* have been reported in theca cells but not granulosa cells during growth of bovine follicles (Stewart *et al.* 1996, Armstrong *et al.* 2000, Llewellyn *et al.* 2007). In addition to IGF1, IGF2 is found in equine follicular fluid (Bridges *et al.* 2002) and can bind and activate IGF1R (Spicer & Aad 2007). IGF2 activity is negatively regulated by IGF2 binding to a second receptor, IGF2R (Delaine *et al.* 2007). Follicular availability of IGF1 is thought to be regulated primarily by IGF-binding proteins (IGFBPs) which render IGF1 inactive (Mazerbourg *et al.* 2003). In ovulatory mares, levels of IGFBPs, most notably IGFBP2 and IGFBP5, decrease in the dominant follicle and this is attributable, at least in part, to the proteolytic activity of pregnancy-associated plasma protein-A (PAPPA, Gerard & Monget 1998, Gerard *et al.* 2004).

The decisive role of IGF1 in follicle selection and the development of ovulatory follicles in mares has been demonstrated in a series of studies by Ginther and associates who showed that injection of IGF1 into the second largest follicle of a wave at the beginning of deviation resulted in the follicle becoming dominant, rather than naturally subordinate, and eventually ovulating (Ginther *et al.* 2004b, 2004c), whereas intrafollicular injection of IGFBP3 blocked the development of the future dominant follicle (Ginther *et al.* 2004a). These responses were associated with specific changes in levels of inhibin A and other growth factors in response to intrafollicular injection of IGF1 or IGFBP3.

In relation to seasonally anovulatory mares, Acosta *et al.* (2004) reported lower concentrations of free IGF1 (not bound to IGFBPs) in large transitional follicles than in ovulatory follicles and Watson *et al.* (2004a) demonstrated that the large transitional follicles had higher levels of IGFBP2. Taken, together, these findings strongly suggest that dominant follicles during transition grow in an IGF1-deficient environment that determines the anovulatory status of transitional mares. Therefore, artificially increasing IGF1 early during the development of a dominant follicle (i.e. before it begins to deviate in diameter from smaller follicles) may be potentially used to facilitate ovulation during transition, as has been reported during the ovulatory season (Ginther *et al.* 2004b, 2004c). Such approach would only be effective if these follicles maintained their response to IGF1 during the transitional period.

The objective of this study was to investigate seasonal changes in follicular responsiveness to IGF1 in mares by testing the hypothesis that the response to intrafollicular injection of IGF1 before the beginning of diameter deviation would be similar during transitional and ovulatory periods. This was done by determining during these two periods 1) granulosa cell expression of *IGF1R* as well as other genes that may have an effect on the responsiveness of cells to IGF1, namely, *IGF2R*, *FSHR*, *LHCGR* and *PAPPA*, 2) follicular levels of IGFBP2 and IGFBP5 and 3) changes in follicular inhibin A and oestradiol levels after an intrafollicular injection of IGF1. In testing the hypothesis, a novel experimental approach was applied using the second largest follicle of an ovulatory wave as a model to study the responses of transitional follicles to injection of IGF1. The rationale for this was based on 1) the common anovulatory nature of these two types of follicles which is derived, at least in part, from their development in a IGF1-deficient environment and 2) the well-established biochemical and functional responses of the second largest follicle of an ovulatory wave to IGF1 injection (Ginther *et al.* 2004c, 2005).

Results

The first ovulation of the year occurred between 11 April and 24 May (median date, 29 April) which coincides with the expected onset of the ovulatory season in this latitude (Watson *et al.* 2004a).

Gene expression in granulosa cells

Granulosa cell pellets for gene expression analyses were obtained from a total of 12 follicle ablation sessions during the transitional period and 14 sessions during the ovulatory period. Between 4 and 7 pools (one to four follicles per pool) were obtained within the 15–24 and 25–34 mm categories during each period. Mean diameter of follicles included in a pool during transitional and ovulatory periods were 19.2 ± 2.0 and 16.9 ± 1.0 mm respectively for the 15–24 mm category, and 29.7 ± 0.3 and 31.7 ± 1.1 mm for the 25–34 mm category. Mean diameters were not different ($P > 0.1$) between periods within each category.

Results of qPCR analyses are shown in Fig. 1. Although there were no main effects ($P > 0.4$) of period or follicle size for *IGF1R* expression, there was a significant ($P = 0.03$) interaction due to sixfold higher ($P = 0.01$) mean levels of *IGF1R* mRNA in 25–34 mm follicles during the ovulatory period than during the transitional period but no differences ($P > 0.5$) between 15–24 mm follicles during the two periods. Analysis within period indicated that during the ovulatory period 25–34 mm follicles had greater ($P = 0.04$) *IGF1R* mRNA levels than 15–24 mm follicles. Although there was no effect of

Figure 1 Relative mRNA levels (mean $\pm$ S.E.M.) of (a) *IGF1R*, (b) *IGF2R*, (c) *FSHR*, (d) *LHCGR* and (e) *PAPPa* in granulosa cells collected from pools of 15–24 and 25–34 mm follicles (one to four follicles per pool) during the transitional period (dashed line, open circle; $n=7$ and 4 pools respectively) and the ovulatory period (solid line, closed circle; $n=6$ and 6 pools). The interaction between follicle size and period was significant for *IGF1R* ($P=0.03$) and *FSHR* ($P=0.007$) and approached significance for *LHCGR* ($P=0.09$), whereas an effect of period for *IGF2R* tended to be significant ($P=0.06$). An asterisk indicates a significant difference ($P < 0.05$) between periods within follicle size. Different letters (a,b) indicate a significant difference ($P < 0.05$) between follicles sizes within period.

follicle size or an interaction ($P > 0.2$) for *IGF2R* expression, the effect of period tended to be significant ($P=0.06$), with higher mean expression levels during the transitional period than during the ovulatory period. As for *IGF1R*, there were no main effects ($P > 0.2$) of period or follicle size for *FSHR* expression but there was an interaction ($P=0.007$) which was due to higher levels (approximately fivefold) in 25–34 mm follicles during the ovulatory period than during the transitional period. In addition, during the transitional period, 15–24 mm follicles had greater ($P=0.024$) levels of *FSHR* mRNA than 25–34 mm follicles. For *LHCGR* expression, the interaction between period and follicle size approached significance ($P=0.09$) due to higher mean expression in 25–34 mm follicles during the ovulatory period than during the transitional period. There were no overall main effects or an interaction ($P > 0.2$) for *PAPPa* expression; however, analysis of expression levels within the ovulatory period indicated a tendency ($P=0.08$) for higher *PAPPa* mRNA levels in 25–34 than 15–24 mm follicles.

Follicular responses to IGF1

Data from a total of seven mares were removed from the analyses after follicular injections for the following reasons; target follicle could not be identified with total certainty (three mares), failed intrafollicular injection (one mare), follicle collapsed after injection with vehicle (one mare) and follicular fluid was grossly contaminated

with blood (two mares). As a result, data from five to seven mares were available for analyses within each treatment and period.

Diameters of target follicles on the day of injection were not affected by period ($P > 0.1$) and were similar ($P > 0.1$) between vehicle- and IGF1-treated mares during the transitional period (largest follicle, 19.2 ± 0.3 and 19.1 ± 0.5 mm respectively) and the ovulatory period (second largest follicle, 20.4 ± 1.2 and 19.8 ± 0.3 mm). Changes in diameter during the first 24 h after injection of vehicle or IGF1 were 0.6 ± 1.3 and -0.3 ± 1.4 mm respectively for the largest follicle during the transitional period, and -2.0 ± 2.1 and -1.6 ± 1.5 mm for the second largest follicle during the ovulatory period. These changes were not affected ($P > 0.1$) by period, treatment or their interaction.

As expected, free IGF1 concentrations in follicular fluid (Fig. 2a) increased after IGF1 injection (main effect of treatment, $P=0.01$) but were not affected by period or the interaction of treatment $\times$ period ($P > 0.1$). Determination of relative IGFBP2 and IGFBP5 levels in follicular fluid by immunoblotting revealed bands that were 32–36 kDa (Fig. 2b) and 25–29 kDa (Fig. 2c) respectively, which is consistent with previous reports using western ligand blotting in equine follicular fluid (Gerard & Monget 1998, Bridges *et al.* 2002, Watson *et al.* 2004a). Follicular IGFBP2 levels were not affected ($P > 0.1$) by period, treatment or period $\times$ treatment but this interaction was significant ($P < 0.03$) for levels of IGFBP5 due to higher levels in vehicle-treated mares

Figure 2 Relative follicular fluid levels (mean $\pm$ S.E.M.) of (a) free IGF1, (b) IGFBP2 and (c) IGFBP5 in mares ($n=5-6$ /group) 24 h after injection of vehicle (white bars) or 10 μ g IGF1 (black bars) into the largest follicle of a wave during the transitional period or the second largest follicle during the ovulatory period. There was an effect of treatment for free IGF1 ($P=0.01$). There were no main effects or an interaction for IGFBP2 ($P>0.1$) but there was an interaction of period $\times$ treatment ($P=0.027$) for IGFBP5. Means with different letters (a,b) are different ($P<0.05$). Representative immunoblots for each IGFBP are shown above the corresponding graph.

during the ovulatory period than in IGF1-treated mares during the ovulatory period ($P<0.05$) and vehicle-treated mares during the transitional period ($P<0.05$).

Follicular inhibin A concentrations (Fig. 3a) were affected by treatment ($P=0.01$) but not by period or treatment $\times$ period ($P>0.1$). Injection of IGF1 induced mean increases ($P=0.05$) in inhibin A levels of 2.1- and 2.2-fold during the transitional and ovulatory periods respectively.

Finally, although there was no overall effect ($P>0.4$) of treatment or an interaction, oestradiol levels in follicular fluid were affected by period ($P=0.05$) resulting in mean higher oestradiol levels (1.8-fold) during the ovulatory period than during the transitional period (Fig. 3b).

Discussion

This study used two complementary approaches in mares to investigate for the first time *in vivo* seasonal changes in follicular responses to IGF1, namely, 1) analyses of follicular *IGF1R* expression and IGFBP levels, both of which are major determinants of responsiveness to IGF1 and 2) quantification of actual follicle responses to intrafollicular injection of 10 μ g IGF1, as indicated by changes in follicular inhibin A and oestradiol levels. The use of the second largest follicle of an ovulatory wave as a reference to study the responses of transitional follicles is novel. Unlike the largest follicle of ovulatory waves (future dominant follicle), the second largest follicle is not exposed to an increase in follicular IGF1 at the

beginning of deviation (Donadeu & Ginther 2002b), which contributes to its inability to become ovulatory (Ginther *et al.* 2004b, 2004c). Transitional follicles develop in a similar IGF1-deficient environment after

Figure 3 Mean ($\pm$ S.E.M.) follicular fluid concentrations of (a) inhibin A and (b) oestradiol in mares ($n=5-7$ /group) 24 h after injection of vehicle (white bars) or 10 μ g IGF1 (black bars) into the largest follicle of a wave during the transitional period or the second largest follicle during the ovulatory period. There were significant effects of treatment for inhibin A ($P=0.01$) and period for oestradiol ($P=0.05$). Means within periods with different letters (a,b) are different ($P<0.05$).

deviation resulting in the developmental deficiencies that prevent them from acquiring the ability to ovulate (Acosta *et al.* 2004, Watson *et al.* 2004a). The present study demonstrates that experimental comparisons between these two types of follicles can be useful to understand the biological mechanisms behind the anovulatory nature of transitional follicles and opens the way for the potential use of this comparative approach in future studies.

To our knowledge, changes in IGF receptor expression during follicular development have not previously been reported in the horse. *IGF1R* expression increased with follicle size during the ovulatory period but not during the transitional period, and this resulted in higher expression levels in 25–34 mm follicles during the ovulatory period than during the transitional period but similar levels in 15–24 mm follicles between the two periods. This result is consistent with the essential role of IGF1 in the selection and subsequent development of ovulatory follicles (reviewed in Beg & Ginther 2006) and also with the causative role of deficient IGF1 stimulation in the failure of transitional follicles to develop characteristics of ovulatory follicles (reviewed in Donadeu & Watson 2007). The present results in mares are in contrast to some of the previous studies in which *IGF1R* expression in granulosa cells did not change (Armstrong *et al.* 2000, Liu *et al.* 2000, Hastie & Haresign 2006, Llewellyn *et al.* 2007) or decreased (Perks *et al.* 1995) during growth of antral follicles in cows and pigs or between seasons in sheep. Taken together, these results highlight species-specific differences in the pattern of *IGF1R* expression during follicle growth; however, it must also be recognised that quantification of mRNA levels in the present and previous studies may not adequately reflect the actual levels of functional IGF1R in follicles.

The expression levels of *IGF2R*, *FSHR* and *LHCGR* in granulosa cells were determined because the responses of follicular cells to IGF1 may depend, at least in part, on their concurrent responsiveness to IGF2 (Spicer & Aad 2007) and gonadotrophins (Glister *et al.* 2001). IGF2 is naturally produced in equine follicular cells (Davidson *et al.* 2002, Watson *et al.* 2004a) and *IGF2* expression is lower during the transitional period than during the ovulatory season (Watson *et al.* 2004a). IGF2 has been shown to promote bovine granulosa cell proliferation and steroidogenesis by binding to IGF1R (Spicer & Aad 2007). In contrast, IGF2 binding to IGF2R results in the inactivation of IGF2 (Delaine *et al.* 2007). In this study, expression of *IGF2R* did not change significantly with follicle size but was higher during the transitional period than during the ovulatory period. Considering the role of IGF2R in regulating IGF2 bioavailability in the follicle, the present results suggest that the ability of IGF2 to activate IGF1R may be impaired in transitional follicles relative to ovulatory follicles. Although the effects of IGF1 injection on *IGF2R* were not determined in this

study, *IGF2R* expression in bovine granulosa cells has been shown to decrease in response to IGF1 (Spicer & Aad 2007).

The effects of season on follicular *FSHR* expression in horses have not been previously reported. In an earlier study, Fay & Douglas (1987) failed to demonstrate changes in FSH binding to follicle walls during the oestrus cycle and those findings are consistent with the absence of significant changes in *FSHR* expression between follicles 15–24 and 25–34 mm during the ovulatory period in the present study. In contrast, *FSHR* expression decreased as transitional follicles grew above 24 mm. The reason for this is not known but it does bring the possibility that a reduced responsiveness not only to LH but also to FSH may be involved in the failure of these follicles to acquire ovulatory capacity. The higher *LHCGR* expression in dominant-size follicles during the ovulatory period than during the transitional period is consistent with previous results (Watson *et al.* 2004b). Although not critically tested in the present study, reduced *LHCGR* expression in transitional follicles is likely mediated, at least in part, by low levels of IGF1 in these follicles (Hirakawa *et al.* 1999). Overall, the results on receptor expression in this study indicated that follicles 15–24 mm have similar responsiveness to trophic stimuli, namely, IGF1, FSH and LH, during transitional and ovulatory periods. Further analyses of IGF1 responsiveness of these follicles between periods were then performed by analysing follicular IGFBP levels and changes in inhibin A and oestradiol in response to intrafollicular IGF1 administration.

There was no effect of period on intrafollicular levels of free IGF1 and this is consistent with results indicating that differences in intrafollicular levels of free IGF1 between transitional and ovulatory waves do not occur before the beginning of deviation (Acosta *et al.* 2004, Donadeu 2006). During the two periods, mean follicular levels of free IGF1 in mares injected with IGF1 or vehicle were lower than those reported in some of the previous equine studies (Donadeu & Ginther 2002a, 2002b, Ginther *et al.* 2004a) but were comparable with natural levels in dominant and largest subordinate follicles respectively, at the beginning of deviation during ovulatory waves in another study (Ginther *et al.* 2005). The magnitude of the increase in intrafollicular free IGF1 after injection of 10 µg IGF1 (average over the periods, 23.2-fold over saline-treated follicles) was consistent with results of previous IGF1 injection studies in ovulatory mares (Ginther *et al.* 2004c, 2005).

In the present study, vehicle-treated follicles were used to assess seasonal differences in the levels of IGFBP2 and IGFBP5, both of which critically contribute to the differential increase in IGF1 availability to the dominant follicle in ovulatory mares (Gerard & Monget 1998, Bridges *et al.* 2002, Donadeu & Ginther 2002b). The follicular puncture procedure used in this study has by

itself no detectable effects on levels of follicular factors, including IGFBPs (Ginther *et al.* 2004c). Follicular IGFBP2 levels were similar between the transitional period (mean diameter of the largest follicle at the time of sampling, 19.5 mm) and the ovulatory period (mean diameter of the second largest follicle, 18.8 mm). In agreement with the present results, IGFBP2 levels were not different between 13 mm follicles collected during the transitional period and the ovulatory season (Donadeu 2006). In contrast, large follicles (>30 mm) had relatively higher IGFBP2 levels during the transitional period (Watson *et al.* 2004a). Taken together, these findings suggest that, in contrast to the ovulatory season (Gerard & Monget 1998, Bridges *et al.* 2002), a decrease in follicular IGFBP2 levels in dominant follicles does not occur during the transitional period. A tendency for greater mean expression levels of *PAPPA*, an IGFBP protease (Gerard *et al.* 2004), in follicles 25–34 mm than in follicles 15–24 mm during the ovulatory period but not during the transitional period in this study are consistent with that conclusion. The lack of an effect of IGF1 injection on follicular IGFBP2 levels is in agreement with results by Ginther *et al.* (2004c, 2005) who showed that a much higher dose of IGF1 than the one used in the present study is needed to induce any detectable changes in follicular levels of IGFBP2.

The effects of season or IGF1 on follicular IGFBP5 levels in mares have not been reported before. The reason for higher levels of IGFBP5 during the ovulatory period (second largest follicle) than during the transitional period (largest follicle) in vehicle-treated mares is unknown. IGFBP5 levels (as well as levels of IGFBP2 and IGFBP4) have been shown to increase in subordinate follicles of ovulatory mares (Gerard & Monget 1998, Donadeu & Ginther 2002b). Based on results in cattle and horses, changes in follicular fluid levels of IGFBP5 and IGFBP4 during follicular waves seem to occur before the beginning of diameter deviation (reviewed in Beg & Ginther 2006). In contrast, changes in follicular IGFBP2 levels are only detectable after deviation. Therefore, it is conceivable that in the present study, IGFBP5 levels (but not IGFBP2 levels) in the target follicle of the ovulatory period (second largest follicle) were already increasing when follicular fluid was collected for analyses. Another observation in the present study was that IGFBP5 levels were lower in IGF1- than vehicle-injected follicles during the ovulatory period. Follicular levels of IGF1 and IGFBP5 are negatively correlated during follicular growth in mares (Bridges *et al.* 2002). Considering that, together with the apparent early involvement of IGFBP5, compared with IGFBP2, during follicle selection (Beg & Ginther 2006), it is plausible that in the present study IGFBP5 levels in the second largest follicle during the ovulatory period decreased in response to exogenous IGF1, a possibility that warrants further testing. Overall, these results on IGFBPs suggest that follicular IGFBP activity may be comparable during the transitional and

ovulatory periods (based on IGFBP2 levels) or lower during the transitional period (based on IGFBP5). Although the relative contribution of IGFBP2 and IGFBP5 to regulation of follicular IGF1 activity in mares has not been directly determined, previous results using western ligand blotting suggest that follicular IGFBP2 is predominant over IGFBP4 and IGFBP5 during both the ovulatory and transitional periods (Gerard & Monget 1998, Watson *et al.* 2004a). Considering this, overall IGFBP activity within follicles before the beginning of deviation is likely to be similar during transitional waves and ovulatory waves and this is consistent with similar levels of free IGF1 after IGF1 injection during the two periods in this study.

Inhibin A levels have been consistently shown to increase after IGF1 injection into the second largest follicle of ovulatory waves (Ginther *et al.* 2004c, 2005) as well as in the largest follicle during natural follicle selection (Donadeu & Ginther 2002b). The mean increase in intrafollicular inhibin A induced by 10 µg IGF1 during the ovulatory period (about twofold relative to vehicle-treated controls) was comparable with the increase reported after IGF1 injection into the second largest follicle in a previous study (Ginther *et al.* 2004c). More importantly, the inhibin A responses were similar during transitional and ovulatory periods and this was consistent with the absence of detectable differences in expression of *IGF1R* and follicular levels of IGFBP2 before the beginning of deviation between the two periods.

The differences in follicular oestradiol levels between periods in the present study indicate that deficient follicular steroidogenesis during transition is not limited to late dominant follicles (Davis & Sharp 1991, Watson *et al.* 2002, 2004b) but it apparently also involves follicles before the beginning of deviation. Since IGF1 is known to stimulate steroidogenesis *in vitro* (Glister *et al.* 2001) and there is a temporal association between reduced IGF1 activity and low oestradiol levels during transition (reviewed in Donadeu & Watson 2007), we wished to examine whether experimentally increasing intrafollicular IGF1 activity before the beginning of deviation during the transitional period would lead to an stimulation of oestradiol production. The absence of an oestradiol response to IGF1 in that regard is similar to results after intrafollicular IGF1 injection before the beginning of deviation in ovulatory mares (Ginther *et al.* 2004c, 2005) and is consistent with results *in vitro* showing that the ability of granulosa cells to respond with an increase in oestradiol may depend on the size of the follicles (Davidson *et al.* 2002). The present result indicates that, as during the ovulatory season, IGF1 may not acutely regulate oestradiol during the transitional period. It is also possible that a higher dose of IGF1 than the one used in this study was necessary to adequately stimulate not only granulosa cells but also theca cells, which is likely required to efficiently stimulate the

natural increase in follicular production of oestradiol at the end of the transitional period (Watson *et al.* 2004b). Nonetheless, the present results together with those of previous studies (Donadeu & Watson 2007) strongly suggest that the distinct increase in production of oestradiol by dominant follicles at the end of the transitional period involves not only an increase in IGF1 bioactivity but also an increase in the responsiveness of follicles to IGF1 and gonadotrophins (and likely other trophic factors).

In summary, expression of *IGF1R*, as well as *FSHR* and *LHCGR*, in granulosa cells from follicles 15–24 mm was similar during the transitional period and the ovulatory period, whereas expression of each of these receptors in follicles 25–34 mm was lower during the transitional period. *IGF2R* expression tended to be higher during the transitional period than during the ovulatory period, regardless of follicle diameter. In addition, follicular levels of IGFBP2 before the beginning of deviation were similar during the two periods whereas levels of IGFBP5 were lower during the transitional period. Injection of IGF1 into the largest follicle of a transitional wave before the beginning of deviation induced a approximately twofold mean increase in follicular inhibin A levels, similar to that induced after IGF1 injection into the second largest follicle of a wave during the ovulatory period. Finally, follicular oestradiol levels did not respond to IGF1 during any of the two periods. Taken together, these results indicate that follicular responsiveness to IGF1 before the beginning of deviation is comparable during transitional waves and ovulatory waves and, therefore, this is likely not a primary cause of the deficient development of dominant follicles during transition.

Materials and Methods

Animals and experimental design

Eight horse mares of mixed breeding, aged 4–16 years, body weight 400–600 kg, were kept under natural light in an open shelter and outdoor paddock in the northern hemisphere (55° N; Edinburgh, UK). Mares were fed alfalfa/grass hay and had free access to water and mineralised salt. All experimental procedures were carried out under the UK Home Office Animals (Scientific Procedures) Act 1986, after approval by the Ethical Review Committee, University of Edinburgh.

Beginning in February, each of the eight mares was used during two different reproductive periods, transitional (February to April) and ovulatory (June to August). Within each period, a cross-over design was used whereby each of these mares was randomly assigned once to each of two treatments, IGF1 and vehicle, leaving a ≥ 30 -day rest between the two treatments. Therefore, the same mares were used within each treatment during the two periods. At the beginning of the transitional period, mares had at least one follicle > 20 mm and no detectable corpus luteum for the previous 4 weeks; these criteria excluded animals that were in deep anoestrus or ovulatory respectively.

Follicle ablation, treatments and sample collection

Before each treatment during both periods, all follicles > 10 mm were ablated by ultrasound-guided transvaginal aspiration of follicular contents, as described (Gastal *et al.* 1997), using a 17 gauge, 55 cm ovum pickup needle (Popper & sons, Inc., New Hyde Park, NY, USA) attached to a 5 MHz curved array transducer on an Aloka 500 V ultrasound scanner (BCF Technology, Livingston, UK). This was done to eliminate follicles from previous waves and provide a uniform follicle status before the treatments across periods. During the ovulatory period, follicle ablations were always performed during mid-cycle (10–15 days after ovulation). In both periods, aspirates from follicles 15–24 and 25–34 mm were collected and separately pooled within each mare. These two diameter categories correspond to follicles before and after the beginning of diameter deviation during a follicular wave (Donadeu & Ginther 2004). Follicle aspirates were centrifuged at 2000 *g* at 4 °C for 5 min and Trizol reagent was added to the granulosa cell pellets before they were frozen at -80 °C for subsequent RNA extraction and gene expression analyses. Cell pellets with gross blood contamination were discarded.

Following ablation of all follicles > 10 mm, the ovaries were monitored daily with the same scanner equipped with a 5 MHz linear-array transducer (BCF Technology). At each scanning session, the diameter of follicles 10–15 mm was estimated by comparison with the graduation marks on the scanner screen and follicles > 15 mm were measured with the electronic callipers. Follicles were measured in two planes and the average of length and width from a frozen image was taken as the actual diameter. Follicles that refilled with fluid to > 15 mm were re-ablated.

During the transitional period, once the largest follicle of the post-ablation wave reached ≥ 18 mm, corresponding to about 1 day before the expected beginning of deviation (Donadeu & Ginther 2004), the follicle was injected with 10 μ g recombinant human IGF1 (NHPP, Torrance, CA, USA) in a 100 μ l citrate buffer or with 100 μ l citrate buffer vehicle. The dose of IGF1 was chosen based on the results of a previous dose-response study in ovulatory mares (Ginther *et al.* 2004c); this dose was intermediate between two doses (2.5 and 25 μ g) that when injected into subordinate follicles induced responses over a 48-h period that were similar to those naturally occurring in dominant follicles. Injection was done by ultrasound-guided transvaginal follicular puncture using an outer 20 g needle to penetrate the ovarian stroma and an inner 25 g needle to enter the follicle, as described (Gastal *et al.* 1995). During the ovulatory period, the second largest follicle of the post-ablation wave was injected once the largest follicle reached ≥ 21 mm, which corresponded to an expected mean diameter for the target follicle (second largest follicle) of 18 mm (Donadeu & Ginther 2004), comparable with the diameter of target follicles during the transitional period. During both the transitional period and the ovulatory period, follicular contents were aspirated from the target follicles 24 h after injection, followed by centrifugation and storage of the resulting supernatant at -20 °C. Follicular aspirates grossly contaminated with blood were discarded ($n=2$ samples). Mares were scanned twice a week between treatments and the presence of a corpus luteum was used to indicate that ovulation had occurred.

Quantification of gene expression by qPCR

Total RNA from pooled granulosa cell samples was isolated using the guanidium isothiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987) and reverse transcribed using Superscript III (Invitrogen) and random primers (p(dN)₆; Promega). The cDNA product was quantified using the SYBR GreenER qPCR SuperMix Universal Kit (Invitrogen) for amplicon detection and ROX as an internal reference dye in a DNA Engine Opticon 2 (MJ Research Inc., Waltham, MA, USA). Primers (Table 1) were designed based on available equine sequences or, in the case of *IGF1R*, based on conserved sequences in bovine, porcine and murine. Primers were designed to span two different exons within the corresponding genomic sequence and their specificity was confirmed by ensuring each product yielded the expected length on an agarose gel. PCR settings used were for all genes analysed 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 57 °C (*IGF1R*, *IGF2R* and *PAPPA*) or 52 °C (*FSHR*, *LHCGR* and *18S RNA*) for 30 s and 72 °C for 45 s. The abundance of each target mRNA was calculated with Mx3000P real-time PCR system analysis software (Stratagene, La Jolla, CA, USA) relative to a standard curve constructed from granulosa cells pooled from equine follicles of different sizes. *18S RNA* was used as internal control for each of the genes analysed. Melting curves for each sample were examined to further confirm the specificity of the qPCR product. Intra-assay coefficients of variation (CV) for all genes analysed were between 0.95 and 3.44%.

IGFBP immunoblotting

Follicular fluid was assayed for protein content using the DC protein assay kit (Bio-Rad). Forty micrograms of protein (corresponding to 6–8 µl of a 1:10 dilution of follicular fluid) were heat-denatured in buffer containing 12% SDS, 40% glycerol, 30% β-mercaptoethanol, 300 mM dithiothreitol, 120 mM EDTA, 1 mg/ml bromophenol blue and 375 mM Tris-HCl (pH 6.8) before being resolved on a 12% polyacrylamide gel. This was followed by electroblotting onto PVDF membranes for 1 h at room temperature. Membranes were then blocked for 2 h in a solution (10 mM Tris, 100 mM NaCl, 0.2% Tween 20) containing 5% BSA and incubated overnight with anti-bovine IGFBP2 (1:1000; 06–107, Upstate, Hampshire, UK) or anti-human IGFBP5 (1:1000; 06–110, Upstate). Membranes were

incubated with HRP-conjugated anti-rabbit anti-immunoglobulin (1:10 000; Amersham Biosciences) for 1 h. After further washing, immune complexes were visualised and quantified using the Super Signal West Femto maximum sensitivity detection system (Pierce Chemical Inc., Rockford, IL, USA) and imaged using Fluro-S Scanning System (Bio-Rad Laboratories Ltd). Densitometry analyses were done using Quantity One Analysis Software (Bio-Rad).

Free IGF1, inhibin A and oestradiol immunoassays

Concentrations of free IGF1 were quantified by a sandwich-type ELISA (Diagnostic Systems Laboratories, Webster, TX, USA) adapted for use with mare follicular fluid (Donadeu & Ginther 2002b). The samples were used undiluted. Anti-sera cross-reactivity with IGF2, insulin or IGFBPs was not found by the manufacturer. Intra-assay CV was 6.5% and assay sensitivity was 25.0 pg/ml. For all assays, sensitivity was considered as 2 standard deviations above the mean optical density (OD) of the zero standard corrected for sample dilution.

Concentrations of inhibin A in the follicular fluid were determined with a sandwich ELISA kit (Diagnostic Systems Laboratories). The kit was developed for use with human samples and was adapted and validated for use with equine follicular fluid in our laboratory. Serial dilutions (1:100 to 1:100 000) of a pool of equine follicular fluid in the provided assay diluent resulted in a displacement curve that was parallel to the standard curve provided (0–1 ng/ml). A working dilution of 1:1000 was used for assaying the follicular fluid samples. This was chosen based on the dilution of pooled follicular fluid that resulted in an OD that was central to the range of the standard curve. According to the manufacturer, there was no significant cross-reactivity of the assay with inhibin B, pro-αC inhibin, or activin-A and -B. The intra-assay CV was 6.7% and the sensitivity was 19.7 ng/ml.

Oestradiol levels in follicular fluid were measured by RIA as described (England *et al.* 1981) without previous extraction of samples. The intra-assay CV was 9.6% and the sensitivity was 5.0 pg/ml.

Statistical analyses

Dixon's Q-test was used to identify outliers using $P < 0.05$ as a cut-off value to exclude a sample from subsequent analysis.

Table 1 Primer sequences used in qPCR analyses of equine granulosa cells.

Target	Accession number	Sequences	Product (bp)
<i>IGF1R</i>	X54980.1	For 5'-AAGCTGAGAAGCAGGCAGAG-3' Rev 5'-CGGAGGTTGGAGATGACAGT-3'	265
<i>IGF2R</i>	XM_001491469	For 5'-GTAACGCTAAGCTTTCCTATT ACG-3' Rev 5'-GGTATACCACCGGAAGTTGTA GG-3'	187
<i>FSHR</i> ^a	S70150	For 5'-TCTTTGGCATCAGCACCTAC-3' Rev 5'-AGAAATCCCTGCGGAAGTTC-3'	399
<i>LHCGR</i>	AY464091	For 5'-CCCGGTAAATACCTAAGC-3' Rev 5'-AGTGTCTGTCCTGTTGAA-3'	229
<i>PAPPA</i>	XM_001487931	For 5'-TCACGCCCAATCAAGT-3' Rev 5'-AGGTCACATGCTGATCC-3'	196
<i>18S RNA</i>	AJ311673	For 5'-GGGGAATCAGGGTTCG-3' Rev 5'-GCTGGCACCAGACTTG-3'	209

^aPrimer sequences from Dell'Aquila *et al.* (2004).

Data for each end point were log-transformed before analyses whenever a Kolmogorov–Smirnov test (significance level = 0.01) indicated lack of normality. For all end points, main effects (period, follicle size and/or treatment) and their interactions were analyzed with the General Linear Model (Minitab 15) using a split-plot design with mare as block. To rule out any carry-over effect derived from the repeated use of mares within each period, an additional main effect included in the analyses was whether a treatment was first or second during a period. When a main effect was significant or approached significance, Tukey's test was used to locate differences between means. A probability of <0.05 indicated that a difference was significant and probabilities between >0.05 and <0.1 indicated a difference approaching significance.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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