Serum Inhibin A and B Concentrations During the Menstrual Cycle in Mothers of Spontaneous Dizygotic Twins

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Dizygotic twinning in humans is influenced by genetic factors suggesting inherited variation affects follicle development and predisposes to double ovulations. In a previous study, we conducted a detailed examination of follicle development and variation in hormone concentrations during the menstrual cycle in mothers of DZ twins (MODZT) compared with an age-matched control group of mothers of singletons. We did not detect differences in FSH concentrations between mothers of twins and mothers of singletons. Serum inhibin concentrations were measured by a radioimmunoassay that did not distinguish between dimeric inhibin A and B forms and free inhibin α subunit. We therefore analyzed the samples from this study with specific assays to determine whether concentrations of inhibin A and B were different between MODZT and controls and therefore contribute to the twinning phenotype. There were no significant differences between MODZT with single ovulations and control women in inhibin A and B concentrations during the cycle, including the critical period for the selection of the dominant follicle. These data suggest that the genetic cause of twinning is not associated with changes in FSH concentrations or recognised feedback mechanisms regulating FSH release.

The tendency to conceive spontaneous dizygotic (DZ) twins is a complex trait influenced by genetic and environmental factors. There are important contributions to variation in twinning from both maternal age and family history (Bortolus et al., 1999; Bulmer, 1970). DZ twinning increases four-fold from maternal age 18 to 37 (Bulmer, 1970). Twinning also clusters within families and is under genetic control (Bulmer, 1970; Lewis et al., 1996; Meulemans et al., 1996; White & Wyshak, 1964). Analysis of 6596 families of twins from the Australian Twin Registry found significantly higher frequencies of DZ twins in female relatives, but not male relatives of DZ twins (Lewis et al., 1996). Taken together, the risk to first-degree female relatives is in excess of 2 (Bulmer, 1970; Lewis et al., 1996; Meulemans et al., 1996), comparable with breast cancer (Claus et al., 1991).

Mothers of DZ twins have a higher incidence of spontaneous multiple follicle growth and multiple ovulation (Gilfillan et al., 1996; Martin et al., 1991). Ovarian function is regulated by complex endocrine and paracrine pathways including a central role for follicle-stimulating hormone (FSH) secreted from the pituitary gland. The ovary in turn regulates FSH secretion by a negative feedback mechanism involving both steroid (estradiol and progesterone) and protein (inhibin A and B) hormones (Baird & Smith, 1993).

Current models suggest multiple ovulation is controlled by both concentrations of FSH above some threshold around the time of ovulation and by intra-ovarian factors (Baird, 1983; Baird & Campbell, 1998; Baker & Spears, 1999; Campbell et al., 1995; Macklon & Fauser, 2000; Zeleznik, 2001). FSH is essential for development of the dominant follicle(s) leading to ovulation. At the end of the luteal phase, FSH concentrations rise due to corpus luteum regression and decreased oestrogen output (Baird, 1983; Baker & Spears, 1999; Macklon & Fauser, 2000; Zeleznik, 2001) stimulating the growth of healthy follicles 2–5 mm in diameter. Subsequently, FSH concentrations decline due to ovarian steroid and inhibin negative feedback and most growing follicles become atretic (Zeleznik, 2001). Usually a single follicle escapes atresia by acquiring increased sensitivity to FSH, probably through increased numbers of granulosa cells, acquisition of LH receptors, and increased FSH receptors on granulosa cells (Baird, 1983; Macklon & Fauser, 2000; Zeleznik, 2001). It remains unclear whether the selection of more than one dominant follicle leading to the birth of dizygotic (DZ) twins results from variation in the hypothalamic-pituitary-ovarian axis or from intra-ovarian mechanisms.

Several studies report increased concentrations of follicle-stimulating hormone (FSH) during the menstrual cycle in mothers of DZ twins (Lambalk et al., 1998; Martin et al., 1984; Nylander, 1973). The higher FSH concentration appears to result from an increase in the number of spontaneous FSH pulses without concurrent luteinizing hormone

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(LH) pulses (Lambalk et al., 1998). We previously conducted a detailed study of follicle development and variation in reproductive hormone concentrations during the menstrual cycle in mothers of DZ twins compared with an aged matched control group of mothers of singletons (Gilfillan et al., 1996). This study did not detect differences in FSH concentrations between mothers of twins and mothers of singletons.

Inhibin is a gonadal protein, which in conjunction with ovarian steroids negatively regulates FSH secretion by the pituitary (Baird & Smith, 1993; Vale et al., 1990). Based on localization studies and serum hormone profiles in normal and hormone stimulated women, it is believed that inhibin B is produced by the gonadotropin responsive small antral follicles evident in the very early stages of the menstrual cycle, while inhibin A is primarily produced by the developing follicles (Welt et al., 2001). Lower production of inhibin B by developing follicles in the early follicular phase may lead to a delay in the fall in serum FSH and thus multiple ovulations. Therefore, differences in inhibin B production may contribute to variation in DZ twinning.

In our previous study (Gilfillan et al., 1996), serum inhibin concentrations were measured by a radioimmunoassay that did not distinguish between dimeric inhibin A and B forms and free inhibin α subunit. Mothers of twins may have intrinsically lower production of inhibin B compared to mothers of singletons. The aim of the present study was to measure concentrations of inhibin A and B using specific ELISAs in the samples previously collected to determine whether concentrations of inhibin A and B were different between mothers of DZ twins and controls and therefore contribute to the twinning phenotype.

Subjects and Methods

The study design and subjects have been previously described in detail (Gilfillan et al., 1996). Briefly, 17 mothers of DZ twins and 8 control subjects were recruited. The study was approved by the Monash Medical Centre Human Ethics Committee and all subjects gave informed consent. In the study group all women were mothers of one set of DZ twins and had never been exposed to gonadotropins or clomiphene citrate. Sixteen of these women had a positive family history of twinning and 5 had DZ twins in their own or parents' sibships. The control group comprised eight mothers of singletons. Only two women had a family history of twins, but not in first degree relatives. All women in both groups had regular menstrual cycles and were less than 40 years of age. There were no significant differences between the two groups with respect to age, parity, or body mass index (Gilfillan et al., 1996).

Women were followed through a complete menstrual cycle. Daily blood samples were collected for an average of 37 days per subject starting from the LH peak + 8 days of cycle 1, through cycle 2, and into cycle 3. Transvaginal ultrasound was performed at intervals through cycle 2 using Acuscan XP-10 ultrasound system (Acuscan, San Francisco, CA).

Serum inhibin A (Groome et al., 1994) and B (Groome et al., 1996) were measured using ELISA assays. Inhibin A was determined in terms of the WHO 91/624 human inhibin A reference preparation. The between assay variation was 13.9% from 22 assays. The assay sensitivity was 2pg/ml. Inhibin B standard (Groome et al., 1996) was provided by N. Groome. The between assay variation was 11.4% from 22 assays. The assay sensitivity was 6.5pg/ml. The data for FSH and inhibin A were log transformed and data for inhibin B were square root transformed for analysis. Transformed data were analysed using ANOVA with repeated measures.

Results

As previously reported (Gilfillan et al., 1996) there were no significant differences between the groups in the numbers of follicles > 7 mm in diameter. The number of small follicles (3–6 mm in diameter) remained the same in MODZT throughout the menstrual cycle, but there was a significant decline in small follicles in the control group mid cycle ($p < 0.05$). All women ovulated during the study cycle.

The hormonal data for each subject were organized about reference points of the first day of menstrual bleeding and the mid-cycle LH/FSH surge. Three women from the MODZT group had double ovulations during the cycle under study detected as the presence of two corpora lutea. No double ovulations were recorded in the control group. Data from the three women with double ovulations were excluded from the analysis and are examined individually below.

The changes in FSH concentrations through the cycle previously reported for MODZT with single ovulations and controls (Gilfillan et al., 1996) were not significantly different for the two groups and are shown in Figure 1a. Data for serum concentrations of inhibin A and B are presented in Figures 1b and 1c respectively. Inhibin A concentrations declined before the start of menses (Figure 1b), rose about 6 days before the LH peak and remained high until the end of sampling. Inhibin B concentrations increased shortly before menstruation to reach maximum concentrations about four days after the onset of menses (Figure 1c). There were no significant differences between MODZT with single ovulations and control women in inhibin A and B concentrations during the cycle, including the critical period for the selection of the dominant follicle. There were no differences when comparing the ratios of FSH with inhibin A or B across the menstrual cycle (data not shown).

Data from women with double ovulations were excluded from the main analysis since the development of more than one dominant follicle is likely to influence production of both steroid and protein hormones by the developing follicles. One woman with double ovulations had very high FSH concentrations in the postmenopausal range (Gilfillan et al., 1996). This was associated with lower inhibin B concentrations during the cycle (data not shown) and data from this individual were not included in further analysis. The remaining two women had FSH concentrations in the normal range. The mean and range in concentrations for FSH and inhibins during the early part of the follicular phase were compared with values for women that subsequently had single ovulations (Figure 2). Concentrations of inhibin A and B in the women with double ovulations were not substantially different from

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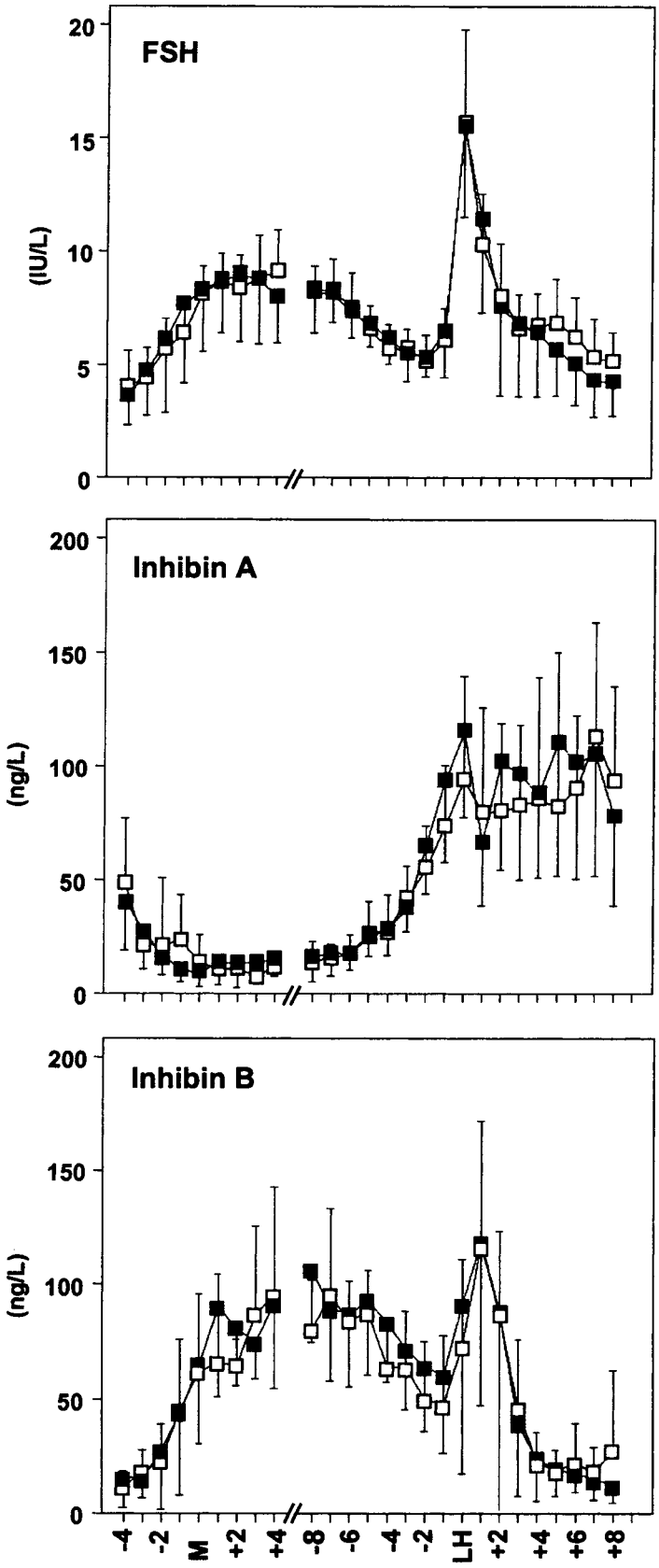

Figure 1

Hormonal profiles for MODZT ($n = 14$) with single ovulations (■) and controls ($n = 8$; □) arranged around the first day of menstruation ($M = 0$) and the day of the LH/FSH surge (LH = 0); (mean ± *SD*).

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either group with single ovulations during this period. There was a tendency for lower inhibin B concentrations in the two women with double ovulations in the early follicular phase, but these were within the range of concentrations in the other two groups.

Discussion

The objective of our original study was to examine changes in hormone concentrations during the cycle in women with a family history of twins, and compare the profiles with those from women with no history of twins in the immediate family. As reported previously, there were no significant differences between MODZT and control groups in the concentrations of FSH during the cycle (Gilfillan et al., 1996) including the height and duration of the increase in FSH over the critical period of follicle selection. In the present study we have extended these observations by measuring the concentrations of inhibin A and B in the two groups. Patterns of inhibin A and B during the cycle were similar to those previously published (Groome et al., 1996; Groome et al., 1994) for women with normal menstrual cycles. In addition, patterns were also similar between MODZT who had single ovulations and the control group with no differences observed at any stage of the cycle, including the period of follicle selection. Three women from the MODZT group had double ovulations during the study. FSH concentrations for one subject were in the postmenopausal range. Hormonal concentrations for the other two were in the normal range, although the concentrations of inhibin B early in the follicular phase were at the lower end of the range for the other two groups.

In agreement with our observations on inhibin B, no differences were detected in inhibin B between MODZT and controls during the early follicular phase in a Dutch study (Lambalk et al., 1998). Our results extend these observations and show no differences in inhibin B across the cycle. Failure to detect differences in inhibin or FSH concentrations in MODZT suggest that clear changes in gonadotropin stimulation and/or hormonal feedback do not underlie variation in twinning frequency in women with a family history of DZ twins. Previous studies have reported elevated FSH concentrations in MODZT (Lambalk et al., 1998; Martin et al., 1984; Nylander, 1973). In particular, a comparison of LH and FSH concentrations between MODZT and controls over a 6 hour period on day three of menstruation reported an increase in FSH concentrations and in the frequency of FSH pulses (Lambalk et al., 1998). It is possible the single daily samples collected in the present study were not frequent enough to detect subtle differences in FSH concentrations responsible for the selection of a second ovulatory follicle. However, no obvious differences in inhibins or FSH were apparent in MODZT in the present study, with or without double ovulations.

Recently, extensive evidence has emerged for paracrine signalling within the ovarian follicle (Erickson & Shimasaki, 2001; Matzuk et al., 2002). Growth factors, including BMP15 and GDF9, secreted by the oocyte, regulate granulosa cell proliferation and differentiation (Dong et al., 1996; Matzuk et al., 2002; Otsuka et al., 2001; Otsuka et al., 2000; Vitt & Hsueh, 2001). This communication between oocytes and somatic cells includes a negative feedback system regulating the influence of BMP15 and kit ligand on granulosa cell mitosis (Otsuka & Shimasaki, 2002). Intra-ovarian signalling pathways regulate coordinated growth of ovarian follicles and the number of ovarian follicles that mature. In the sheep, mutations in two genes from these pathways increases twinning (Galloway et al., 2000; Mulsant et al., 2001; Wilson et al., 2001). Loss of function mutations in the X-linked oocyte-derived growth factor gene BMP15 in two independent lines of sheep cause increased ovulation rate and infertility in a dosage-sensitive manner (Galloway et al., 2000). An activating mutation in the conserved intracellular domain of the receptor BMPR1B is responsible for increased ovulation rate and twinning in sheep carrying the *FecB* allele (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001).

The hypothesis that emerges from these studies is that the oocyte plays a major role in determining the way in which FSH acts in the follicle to regulate granulosa cell differentiation and follicle development (Erickson & Shimasaki, 2001; Matzuk et al., 2002). In sheep carrying the BMPR1B mutation, the primary phenotypic effects occur in the ovary with increased ovulation rate and differences in the size and number of ovulatory follicles (Montgomery et al., 2001; Montgomery et al., 1992). There is also evidence for gene specific effects on FSH release (Montgomery et al., 2001). Some, but not all studies found significantly higher FSH concentrations in homozygous carriers compared with control animals (Montgomery et al., 2001). It remains to be determined how the mutation in BMPR1B influences the release of FSH, but it appears that the effect of the mutation in the ovary is likely to amplify any differences in circulating FSH concentration. In the present experiment, we showed no differences in circulating inhibin or FSH concentrations. The only significant findings of the earlier study were increased progesterone concentrations and an altered LH to progesterone ratio in MODZT with single ovulations suggesting differences in follicle development (Gilfillan et al., 1996). By analogy with effects of the BMPR1B mutation in sheep, mechanisms responsible for control of twinning may reside primarily in the ovary with small primary or secondary effects on circulating FSH concentrations. It is unlikely that DZ twinning is associated with common variants of moderate effect in BMPR1B. No linkage was detected between DZ twinning and markers spanning the region of chromosome 4 containing the BMPR1B locus in the human (Duffy et al., 2001). Variation influencing DZ twinning may lie elsewhere within signaling pathways in the ovary.

In summary, examination of the serum profiles of inhibin A and B in MODZT during a single ovulatory cycle showed no differences when compared to controls. These data suggest that the genetic cause for twinning is not associated with changes in FSH concentrations or the recognized feedback mechanisms involved in regulating FSH release.

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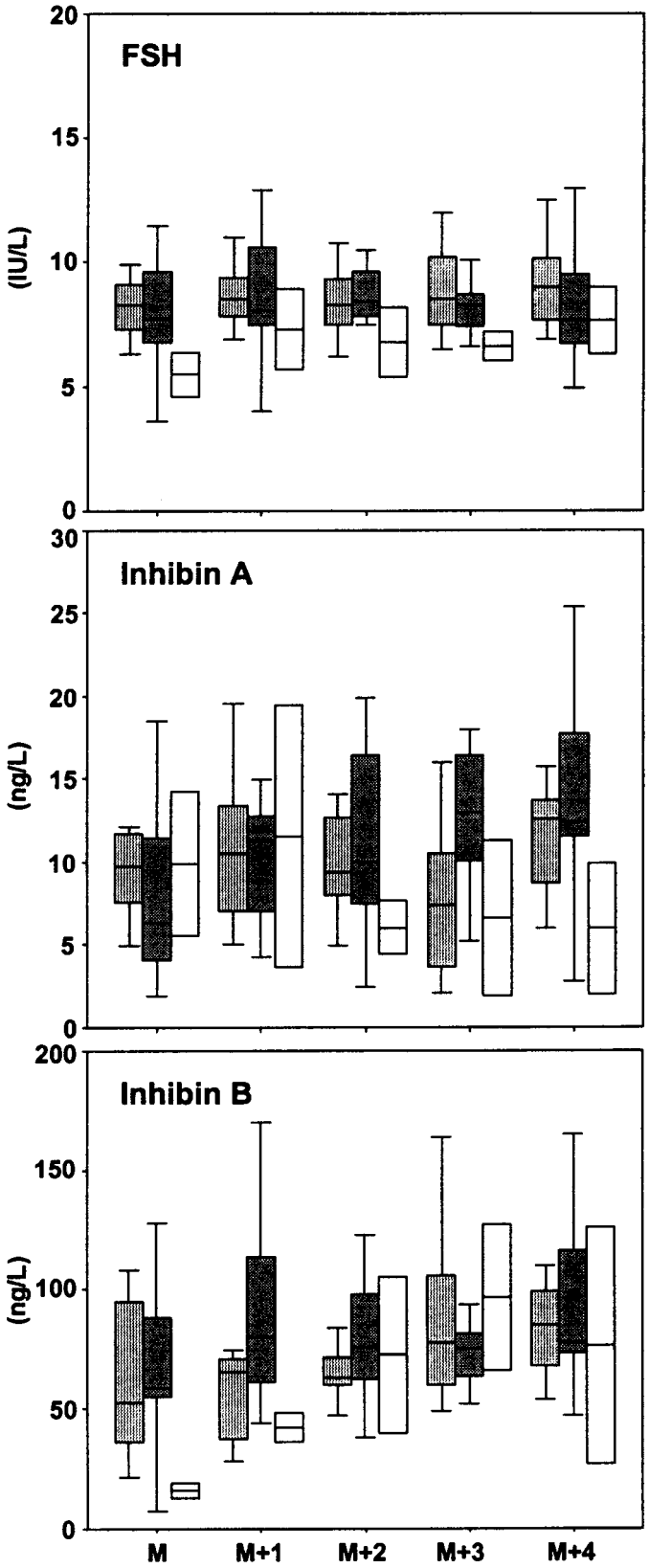

Figure 2

Box plots for the mean and range of hormone concentrations in MODZT (single ovulations; boxes with vertical lines), Controls (black boxes) and MODZT (two ovulations, open boxes) during the early part of the follicular phase of the menstrual cycle.

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