Elevation of follicular phase inhibin and luteinizing hormone levels in mothers of dizygotic twins suggests nonovarian control of human multiple ovulation*

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Objective: To determine whether multiple ovulation in mothers of spontaneous dizygotic (DZ) twins is because of higher hypothalamic stimulation or is in response to lower serum levels of ovarian inhibin.

Design: Serum hormone levels were measured at five times throughout the cycle in a sample of eight mothers of DZ twins and paired controls. On day 12, ovarian ultrasonography was performed.

Setting: Blood samples were collected in participants' homes except on day 12 when they were collected at the ultrasonography clinic.

Patients, Participants: Human volunteers who had at least one set of spontaneous DZ twins were paired with controls matched for age and parity.

Interventions: None.

Main Outcome Measures: Serum inhibin, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) levels on approximate cycle days 1, 2, 8, 12, and 21.

Results: Serum inhibin levels were elevated throughout the cycle (significantly on day 1) in mothers of DZ twins. Also elevated were early follicular FSH levels, LH levels throughout the follicular phase (significantly on days 1, 2, and 8), and early to midfollicular E2 (significantly on day 8) in DZ mothers, indicative overall of greater follicular activity.

Conclusion: It is concluded (1) that the primary cause of multiple ovulation in humans is not a decrease in inhibin secretion from the ovary; (2) the increased secretion of FSH and LH may be caused by elevated secretion of, or sensitivity to gonadotropin-releasing hormone; and (3) the elevated inhibin and E2 levels are a response to increased gonadotropin release. Fertil Steril 56:469, 1991

Dizygotic (DZ) twinning in humans appears to run in families, but otherwise little is known about its etiology.1-3 The increased rate of multiple pregnancies after ovulation induction in anovulatory women4 suggests that the immediate physiological cause of the tendency to multiple births is alteration in the mother's hormone levels, and this is supported by extensive animal data.5 The first evidence that women who gave birth to spontaneous DZ twins might be hormonally unusual was the report of (nonsignificant) midcycle elevations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in mothers of twins.6 However, because follicles are recruited for growth very early in the menstrual cycle,7 or even at the end of the previous cycle,8 it is unlikely that a higher midcycle FSH peak is directly relevant to the cause of multiple ovulation.
In a previous study, we found that FSH and LH levels were elevated in the early follicular phase of mothers who had had two (natural) sets of DZ twins compared with matched controls.\(^9\) We also found elevated midfollicular estradiol (E\(_2\)) levels, presumably reflecting increased follicular activity in mothers of spontaneous DZ twins. We have since obtained direct support for this idea from ovarian ultrasonography in successive cycles of mothers of spontaneous DZ twins and controls.\(^9\)

A key question not addressed in our previous study was whether the elevation of FSH levels in these women is because of greater hypothalamic stimulation or is in response to lower serum levels of ovarian inhibin. We have now repeated this study, measuring serum inhibin, FSH, LH, and E\(_2\) in a new sample of mothers of DZ twins and controls.

**MATERIALS AND METHODS**

**Subjects**

To be included in the study, women had to be having regular, natural cycles, i.e., not menopausal, pregnant or lactating, and not using oral contraceptives or other hormone preparations. Mothers of twins were ascertained through the Australian Multiple Birth Association and mothers-of-twins clubs in Southeastern Queensland and Sydney. There was no overlap with the sample in our previous study.\(^9\) We selected women for whom we had evidence of multiple ovulation on at least two occasions. One woman had had three sets of twins, four women had two twin pregnancies, and three women had one set. These eight women were also taking part in a parallel study\(^10\) to assess the frequency of multiple ovulation in mothers of DZ twins and controls and had had ovarian ultrasound (US) on day 12 in a number of cycles. All had shown evidence of multiple ovulation (at least 2 follicles > 12 mm in diameter) in at least one cycle before that in which hormone levels were measured.

It is crucial to establish that these are indeed mothers of DZ and not monozygotic (MZ) twins. Five mothers had at least one pair of opposite sex (and therefore DZ) twins. Two mothers had pairs with obvious differences in hair and eye color. The twins of the remaining woman were similar in appearance, and it was difficult to make an unequivocal diagnosis of zygosity. In this case, blood samples were taken, deoxyribonucleic acid (DNA) extracted and digested with Hinf III, and DNA fingerprints were obtained by hybridizing the Southern blot with an M13 probe.\(^11\) Several distinct band differences could be seen, and these could be shown to be inherited from the mother and father. One woman had a pair of opposite sex twins and a second pair who appeared identical. This latter pair was subsequently shown by DNA fingerprinting to be MZ.

Controls were selected from women who had had no twin pregnancy themselves and who claimed no family history of twinning. An attempt was made to match each mother of twins to a control as close as possible to her in age and parity.

**Protocol**

Subjects informed us immediately at the onset of menses, and each woman had blood samples collected on 5 days of this cycle: 2 days of the early follicular phase (cycle days 1 and 2 or 2 and 3), 1 day in the midfollicular (about day 8), 1 day in the late follicular phase (about day 12) when ovarian US (transabdominal) was also performed, and 1 day in the midluteal phase (about day 21). For one woman, we missed the first sampling day. Subjects were asked their usual cycle length, and if this differed by more than a day from 28, the last two sampling days were adjusted accordingly.

On each of the 5 sampling days, 4 × 10 mL heparinized intravenous blood samples were drawn at 10-minute intervals (i.e., at 0, 10, 20, and 30 minutes) to reduce sampling error arising from pulsatility in gonadotropin release. Plasma samples were stored at −70°C. Before assaying for inhibin, FSH, and LH, all 320 samples (16 subjects, 5 days, 4 times) were individually randomized, and assays were performed in a single batch. Inhibin assays were made in duplicate for each sample. Because of limited quantities of plasma, not all assays could be performed for some samples, and there are a number of missing values, particularly for LH. For E\(_2\), most remaining plasma samples had to be pooled across some time points within days, but duplicate assays of each pooled sample were performed. For the purposes of analysis, all assays for a given day were treated as replicates, whether duplicates of the same sample or assays of different time points. For each subject/day then, the number of replicates varies, but this is accounted for in the statistical analysis.

**Assays**

Serum inhibin was determined by a specific heterologous inhibin radioimmunoassay (RIA) using an antiseraum raised in rabbits against bovine 31K inhibin and iodinated 31K inhibin as a tracer. The
inhibin standard used was a serum pool obtained from women undergoing ovarian hyperstimulation for in vitro fertilization and embryo transplant. The intra-assay and interassay coefficients of variation (CVs) for eight inhibin assays were 3.7% and 3.3%, respectively.

Serum LH and FSH concentrations were determined by RIA using reagents supplied by the World Health Organization Matched Reagents Program and are expressed in terms of the First International Reference Preparation of LH and the Second International Reference Preparation of FSH immunoassay standards with interassay CVs of 7.3% and 7.8%, respectively. Both serum E2 and progesterone (P) were measured in duplicate by RIA (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA) with interassay CVs of 8.7% and 8.1%, respectively, from 150 assays.

Statistical Analysis

The daily mean for each woman was based on up to four assays for FSH, LH, and E2, and up to eight assays for inhibin. Most means were based on these maximum numbers, but in some cases there was insufficient serum to perform the full set of replicate assays. The data were analyzed by a method of weighted least squares, equivalent to a conventional t-test in which each subject/day mean is weighted by the number of assays on which it is based.

Pooling over all subjects and days, we found highly significant correlations between the 79 subject means and their corresponding variances for inhibin (0.64), FSH (0.32), LH (0.68), and E2 (0.56). For the purposes of statistical analysis, it is desirable to minimize this mean-variance dependency, and this may be achieved by a variance stabilizing transformation. Taking square roots of the raw observations for these four hormones reduced the mean-variance correlations to 0.02, 0.11, 0.31, and 0.23, respectively. A more extreme logarithmic transformation produced correlations of −0.44, −0.04, −0.30, and −0.35. In the interest of simplicity, we chose the square root transformation as the best compromise for analysis of the data. The results (means and SEs) are reported on the original scale.

RESULTS

Mothers of DZ twins had an average of one more pregnancy than controls and were slightly heavier, but these differences were not significant. Table 1 also shows that matching of age was very close and that the mean heights of the two groups were very similar. Ideally, we should have liked to have all subjects under 40, but in fact, five of our subjects (3 mothers of twins, 2 controls) were in their early 40s. Usual length of cycle and number of days of bleeding were similar in the two groups. More follicles ≥ 12 mm in diameter were seen, and there was a greater total follicular volume revealed by ovarian US at about day 12 in the mothers of twins, although these differences did not quite reach significance. Although this result is in line with the finding in our parallel study of greater follicular activity in mothers of twins, it is exaggerated by our failure to observe any follicles in two control women (although their cycles were judged to have been ovulatory).

Before data analysis, the hormonal profiles of each woman (including the day 21 P level), together with the results of the ovarian US scan and information on age and usual cycle length, were evaluated by one of us (H.G.B.) who was blind to the status of the subject as a mother of twins or a control. All 16 cycles were judged to have been ovulatory. One woman (a mother of twins 37 years of age) on days 2 and 3 had FSH levels > 14 IU/L and LH levels > 10 IU/L, and high levels were still apparent at

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Mother of DZ twins (n = 8)</th>
<th>Controls (n = 8)</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>35.9 ± 5.1 (26 to 42)</td>
<td>35.5 ± 5.5 (26 to 43)</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>3.6 ± 6.1 (2 to 6)</td>
<td>1.5 ± 1.5 (1 to 6)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>64.4 ± 7.9 (55 to 78)</td>
<td>165.9 ± 8.8 (155 to 180)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td>59.5 ± 9.3 (51 to 73)</td>
</tr>
<tr>
<td>Usual cycle length (d)</td>
<td>27.3 ± 1.3 (25 to 29)</td>
<td>27.8 ± 2.1 (25 to 30)</td>
</tr>
<tr>
<td>Usual length bleeding (d)</td>
<td>4.9 ± 1.2 (3 to 6)</td>
<td>5.3 ± 1.3 (3 to 7)</td>
</tr>
<tr>
<td>No. of follicles ≥12 mm diameter (day 12)</td>
<td>1.4 ± 1.2 (0 to 4)</td>
<td>0.6 ± 0.5 (0 to 1)</td>
</tr>
<tr>
<td>Total follicular volume (mL on day 12)</td>
<td>4.2 ± 0.0 (0.1 to 13.5)</td>
<td>1.9 ± 1.7 (0.0 to 3.4)</td>
</tr>
<tr>
<td>P (nmol/L on day 21)</td>
<td>32.1 ± 4.0 (16 to 43)</td>
<td>28.2 ± 3.8 (15 to 45)</td>
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*Values are means ± SD with ranges in parentheses.

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Vol. 56, No. 3, September 1991

Martin et al. *Hormone levels in mothers of twins* 471
day 6; 2 years after these data were collected, she was questioned for signs of menopause, but none were apparent. A second mother of twins (33 years old) appeared to have a very short follicular phase (day 6 LH was 10.1 IU/L). Results of both women were included in the analysis.

Results of hormone assays for twin mothers and controls on each study day are shown in Figure 1 and the (two-tailed) significance of differences between means of the two groups is indicated. Serum FSH levels did not differ significantly between the groups on any day. However, serum LH levels were significantly elevated on days 1, 2, and 8 of the cycle. Serum inhibin levels were significantly elevated on day 1 of the cycle, whereas E2 levels were elevated on day 8.

DISCUSSION

Our results replicate most of the findings from our earlier study.9 In that study, we compared FSH, LH, and E2 levels on days 1, 2, 3, and 8 in eight mothers who each had two sets of DZ twins and six controls; we found higher early follicular levels of FSH and LH and higher midfollicular levels of E2 in mothers of twins. In the present study, we obtained hormone assays in a new sample of eight mothers who all had at least one set of DZ twins (3 had 2 sets and 1 had 3 sets) and in whom we had also seen further evidence of double ovulation by ovarian US. These were compared with eight controls matched for age and parity but with no personal or family history of twinning. The new features of this study are that (1) in addition to serum FSH, LH, and E2, we also assayed for serum inhibin on approximate days 1, 2, 8, 12, and 21 and (2) we attempted to establish if multiple ovulation occurred in the cycle under study by using ovarian US to count and measure follicles on approximate cycle day 12.

The present study confirms our previous finding in mothers of twins versus controls of (1) much higher levels of LH throughout the follicular phase and (2) higher levels of E2 in early to midfollicular phase. As in our previous study, these differences were significant for LH on cycle days 1, 2, and 8,
and for E₂ on cycle day 8. The elevation of FSH levels in mothers of twins on days 1 and 2 was not significant in this study, although it was in the previous one.⁹

Two women (both mothers of twins) had more than one follicle ≥ 12 mm in diameter observed at US (1 had 4 follicles > 15 mm diameter). When the analyses were redone omitting their data, the means and significance levels were little changed from those shown in Figure 1, so the differences in group means cannot be attributed to these instances of multiple ovulation in the cycles under study. One of these multiple ovulators was also referred to earlier (see Materials and Methods) as having high follicular FSH and LH levels. If the other woman also reported on for having a short follicular phase is also excluded (for a total of 3 exclusions from the mothers of DZ twins group), most of the features of the results are still retained, although the FSH mean is now lower than controls on days 2 and 8.

Elevated E₂ levels in twin mothers throughout follicular phase are consistent with an increased number of developing follicles secreting estrogen (E),¹⁴ as confirmed in our parallel study using ovarian US at day 12.¹⁰ At least two follicles of at least 10 mm in diameter were seen in three of the eight twin mothers but in none of the controls at the time of the day 12 blood sample. Further confirmation is seen in the correlation of 0.50 between E₂ at day 8 and total follicular volume calculated from the day 12 ovarian US results (data not shown).

A plausible hypothesis arising from our first study, and which was the motive for the present study, is that the elevated gonadotropin levels observed in the early follicular phase of mothers of DZ twins are caused by lower than average inhibin levels. Results of the present study do not support this hypothesis. In fact, inhibin levels are higher in mothers of twins throughout the follicular phase, significantly so on day 1 (P = 0.038) and almost so on day 8 (P = 0.051).

One interpretation of our results focuses on the marked elevation of LH observed in mothers of twins in both our previous study and the present one. This may point to increased levels of (or sensitivity to), gonadotropin-releasing hormone (GnRH) as the primary cause of natural multiple ovulation. This would cause increased levels of both LH and FSH in twin mothers. However, although increased FSH may recruit more follicles (which will release more E as they develop), it also directly stimulates increased production of inhibin resulting in a negative feedback on FSH production.¹⁵⁻¹⁷ Thus, FSH levels would be muted under influence of these hormones. This hypothesis would account for our data and makes the strong prediction that twin-prone mothers will have high early follicular levels of GnRH, that the pituitary has an increased general sensitivity to GnRH, or that the specific regulation by GnRH of LH, FSH, and gonadotropin α-subunit secretion is altered.¹⁸

It should be remembered that our hormone assays are of immunoactivity and not bioactivity. Thus, other considerations that may explain our findings include differences in bioactive FSH between groups, or differences in sensitivity to FSH of the ovarian follicular unit. It is also possible that there are differences in the previous cycle's late luteal phase FSH (which was not examined in this study) that might lead to early recruitment of follicles; in a future study we intend to investigate this.

In conclusion, we suggest that the search for the primary cause of natural multiple ovulation in humans should now shift to those factors that regulate production of, or sensitivity to, GnRH. This is in sharp contrast to multiple ovulation in Booroola merino ewes which appears to have a primary association with low inhibin levels.⁵ The role of other regulators, including activin and follistatin (activin binding protein), also needs to be explored.¹⁹,²⁰ Finally, it must be recognized that there may be several different mechanisms that can cause multiple ovulation in humans, as is becoming apparent in sheep,²¹ and that studies such as the present one confound these.

Acknowledgments. We thank Miss Debrah Redman (Queensland Institute of Medical Research) for coordinating the study; Michael Cervenak, M.B., B.S., and Peter Ryan, M.B., B.S., (South Coast Radiology, Southport, Queensland), Grant Withey, M.B., B.S., (Royal Women’s Hospital, Brisbane), Bernard Chaitowitz, M.B., B.S., (Campbelltown, New South Wales) and David Dye, D.M.U., (Nambour General Hospital, Nambour, Queensland) for performing US scans; Sister Margaret Payne, R.N., Sister Ngaire Knight, R.N., Sister Margaret McJannett, R.N., and Sister Jane Palmer, R.N., (all from Queensland Institute of Medical Research) for collecting blood samples; Phil Chen, Ph.D., (Queensland Institute of Medical Research) for advice and Melanie Southall, ADCLT., (Queensland Institute of Medical Research) for assistance in DNA fingerprinting; Mohan Bangah, B.Sc., (Prince Henry’s Hospital, Melbourne) and Anne O’Connor, B.Sc., (Department of Anatomy, Monash University, Melbourne) for assistance with hormone assays; David Handelsman, M.B., B.S., Ph.D., (Department of Medicine, University of Sydney) for discussion on the design of the study; Sue Healey, B.Sc., (Queensland Institute of Medical Research) for preparing figures and anonymous referees for helpful comments. Most of all we thank the mothers who took part so willingly and made this study possible.

Martin et al. Hormone levels in mothers of twins 473

Vol. 56, No. 3, September 1991
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